University of Alberta

# A Systematic Approach to the Encapsulation of Islets for Transplantation

by

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## ABSTRACT

Islet transplantation has been shown to be an attractive prospect in the treatment of patients affected by type 1 diabetes. However, this therapy is limited in its use and applicability. Current cadaveric human sources are insufficient to meet the growing demands for transplantable islets. Also, islet transplantation therapy is limited by the ability of donor tissue to survive and function in immunologically competent recipients. Strategies to prevent rejection which will subsequently allow the reversal of the diabetic state must be developed.

Encapsulation of islets in alginate has been proposed as a means of protecting islets from host immunity. We demonstrate that encapsulation is also beneficial in its ability to provide an environment suitable to the survival and function of the islets. Islets encapsulated in alginate demonstrated a superior ability to survive and function in long term tissue culture over non-encapsulated islets. Furthermore, these encapsulated islets showed greater longevity of function and improved glucose responsiveness when transplanted into immune-compromised recipients.

In response to proposed alginate immunogenicity, we next developed a modification protocol and assessed the changes in alginate properties as a function of transplantation success. Modification resulted in improved survival of syngeneic recipients with improved glucose responsiveness over islets in pre-modification alginate. Immune presence on capsule recovery indicated that purified capsules were less immunogenic and contained islets with demonstrably improved health and function.

Finally, in examining xenogeneic islets, we demonstrated that the capsule provided a measure of protection to the islets, though insufficient for the normalization of blood glucose. Inclusion of immunoprotective Sertoli cells into the capsule provided an increased measure of support, though again this did not translate into an improvement in the function of the graft, at least in immune competent animals.

In summary, we have provided evidence for the use of alginate encapsulation in long term tissue culture of islets. Composition of the alginate used is of critical importance in transplantation success, though the benefit is limited in its scope. Whereas Sertoli cells provide another level of protection, more investigation is needed to ensure the long-term survival of encapsulated islets in an immune competent host.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AN69 | Polyacrylonitrile |
| ANOVA | Analysis of variance |
| AUC | Area under the curve |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| CK | Cytokeratin |
| CSII | Continuous subcutaneous insulin infusion |
| DKA | Diabetic ketoacidosis |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediamine tetra-acetic acid |
| ES | Embryonic stem (cell) |
| F | Fraction |
| FCS | Fetal calf serum |
| G | Guluronate |
| GLUT | Glucose transporter protein |
| HAR | Hyperacute rejection |
| HBSS | Hank's balanced salt solution |
| HEMA-MMA | Hydroxyethyl methylacrylate-methyl methacrylate |
| HLA | Human lymphocyte antigen |
| IDDM | Insulin dependent diabetes mellitus |
| IE | Islet equivalents |
| Ig | Immunoglobulin |

| | |
|---|---|
| IP | Intraperitoneal |
| IP3 | Inositol triphosphate |
| IPTR | International Pancreas Transplant Registry |
| IRS | Insulin receptor substrate |
| M | Mannuronate |
| MAP | Membrane associated protein |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MHC | Major histocompatibility complex |
| mRNA | Messenger riboxynucleic acid |
| NMR | Nuclear magnetic resonance |
| NMWL | Nominal molecular weight limit |
| NPI | Neonatal porcine islet |
| NPSC | Neonatal porcine serum |
| NPS | Neonatal porcine Sertoli cell |
| OGTT | Oral glucose tolerance test |
| PBS | Phosphate buffered saline |
| PC | Prohormone convertase |
| PEG | Polyethylene glycol |
| PERV | Porcine endogenous retrovirus |
| PI-3 | Phosphatidyl inositol 3 |
| PKB | Protein kinase B |
| PLL | Poly-L-lysine |
| PP | Pancreatic polypeptide |

| | |
|---|---|
| RER | Rough endoplasmic reticulum |
| RIA | Radioimmunoassay |
| SEC-MALLS | Size exclusion chromatography multi-angle laser light scattering |
| SEM | Standard error of the mean |
| SI | Stimulation index |
| STZ | Streptozotocin |
| TCA | Tricarboxylic acid |
| TGF-β | Transforming growth factor-β |
| UP-MVG | Ultra-pure medium viscosity high guluronate (alginate) |
| VBAP | Vascularized bioartificial pancreas |
| VEGF | Vascular endothelial growth factor |
| WHO | World Health Organization |
| XNA | Xenoreactive antibodies |
| α1,3-Gal | α1,3-galactose |
| α1,3-GT | α1,3-galactosyl transferase |

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 THE PANCREAS

The name pancreas derives from the Greek *pan creas*, meaning "all flesh" or "tissue throughout". As the name denotes, it is a fleshy organ, lying directly below the large curvature of the stomach that seems to wrap around the duodenum. In humans the pancreas typically weighs between 70-150 grams, measures 15-25 cm in length, and is connected to the duodenum by the ampulla of Vater, where the main pancreatic duct merges with the common bile duct. The pancreas is formed embryologically by two buds originating from the endodermal lining of the duodenum: the dorsal pancreatic bud in the dorsal mesentery and the ventral pancreatic bud forming close to the bile duct. As the duodenum rotates and becomes C-shaped, the ventral bud and hepatopancreatic orifice move dorsally until ventral bud meets with and lies directly below and behind the dorsal bud. Later the parenchyma and vasculature of the dorsal and ventral buds fuse, with the ventral bud forming the uncinate process and inferior part of the head of the pancreas. The remainder of the pancreas derives from dorsal bud. The mature pancreas, while homogeneous, is divided anatomically for descriptive purposes into the head (adjacent to the duodenum), the body (under the greater curvature of the stomach) and the tail (adjacent to the spleen).

The pancreas is an organ made up of two divergent types of glandular tissue: exocrine cells that secrete digestive enzymes into the lumen of the small intestine, and endocrine cells that secrete hormones into the bloodstream. The cells of the exocrine

pancreas are organized into acini and are rich in endoplasmic reticulum, Golgi complex, and secretory granules containing the digestive enzymes, various nucleases, proteases, amylases and lipases. At the junction of the acini and ducts are centroacinar cells that secrete bicarbonate and other non-enzymatic secretions. The smaller ducts empty into larger ones and eventually into the main or ventral pancreatic duct, then into the bile duct and into the small intestine. Secretion of pancreatic juices is controlled both by hormonal and neural stimuli. The cells of the endocrine pancreas are grouped mainly into islets of Langerhans and make up approximately 2% of the total mass of the pancreas (1). These islets are clusters of cells embedded in and scattered throughout the exocrine pancreas, though they tend to be found relatively close to ductal structures (2), and can be up to 1,000,000 in number in the adult human pancreas (3). Furthermore, in addition to the exocrine and endocrine glandular components, the pancreas has a rich arterial blood supply, extensive lymphatic drainage, and abundant sympathetic and parasympathetic nerve supplies.

### 1.1.1 The Islets of Langerhans

In 1869, German medical student Paul Langerhans first described the presence of clusters of cells floating throughout and seemingly unconnected to the acinar cells of the pancreas (4). These islets of Langerhans are roughly spherical in shape and vary in size from about 50 μm to 500 μm in diameter (5). Islets consist of endocrine cells of four main types. The PP-cells are infrequent, making up 1-2% of islet mass. These cells secrete pancreatic polypeptide, the function and control of which is largely a mystery. The δ-cells form 2-8% of the islet mass and secrete somatostatin which functions in a largely paracrine manner to regulate the cellular secretions of other cells in the islet (6). The α-

cells are the second most numerous cells making up about 25% of the islet. The α-cells produce glucagon, which is released in response to insulin and low blood glucose concentrations, and which acts in both a paracrine manner to regulate insulin release, as well as distally to regulate glucose output from the liver. The β-cells are the most numerous cells in the islet, composing 60-80% of the total islet mass. These cells seem to be segregated more to the center of the islet, with other cell types being found more at the periphery, though this phenomenon is less present in humans than in rodents.

### 1.1.2 The β-cell

In 1907, Lane was the first man to describe the β-cell (first called the A-cell) (7). Then, in 1922, Banting and Best published findings that would change the world with the discovery of the hormone isletin (8), now known as insulin. The β-cells are unique in the human body in that they sense changes in circulating blood glucose concentrations and respond to elevations in glucose by releasing insulin. Insulin is stored in the cytoplasm of the β-cell in secretory granule, where the insulin forms crystals with $Zn^{2+}$. The β-cell releases intracellular insulin via exocytosis in response to changes in perfusing blood glucose concentration. Glucose binds to glucose transporter in the cell membrane of the β-cell and is moved into the cell. Once inside the cell, glucose undergoes glycolysis in the cytoplasm followed by the tricarboxylic acid (TCA) cycle in the mitochondrion of the β-cell. Electron transport drives ATP generation, closing $K_{ATP}$ channels leading to depolarization of plasma membrane potential and subsequent opening of voltage-dependent $Ca^{2+}$ channels. Increased calcium levels causes phospolipase C activation, cleaving the membrane phospholipid phosphatidyl inositol 4,5-bisphosphate into inositol 1,4,5-triphosphate and diacylglycerol. Inositol 1,4,5-triphosphate ($IP_3$) binds to receptor

proteins in the membrane of ER, allowing the release of $Ca^{2+}$ from the ER via $IP_3$ gated channels further raising the cell concentrations of calcium. Significantly increased amounts of calcium in the cells causes release of previously synthesized insulin, stored in secretory vesicles. Elevation of cytosolic $Ca^{2+}$ induces fusion of insulin storage granules with the plasma membrane and subsequent insulin exocytosis, as well as up-regulating the insulin gene. Despite the straight forward nature of this response, insulin release from the β-cell is further regulated by various neurotransmitters, peptides and hormones such as glucagon (9). Furthermore, insulin release is characterized by a biphasic response to elevations in blood glucose, with a transient first phase 'burst' of insulin, followed by a second sustained phase lasting for the remainder of the period of glucose stimulation (10). Additionally, insulin secretion is oscillatory under both basal and stimulated steady state conditions (11).

### 1.1.3 Insulin

The insulin gene is specifically expressed in the pancreatic β-cell, and its sequence is remarkably conserved throughout evolution and across species (12). Transcription of the gene yields the mature preproinsulin mRNA 446 bp long. This process is tightly controlled by a number of factors and processes. The preproinsulin peptide is synthesized on the rough endoplasmic reticulum (RER). The signal peptide is cleaved from the final product, releasing the proinsulin peptide into the lumen of the RER. Translation and proinsulin biosynthesis is also controlled by various nutrients, hormones and neurotransmitters (13). Proinsulin is moved from the RER to the Golgi apparatus, then to immature β-granules where the processing of proinsulin to insulin occurs (14). The single-chain polypeptide proinsulin encompasses 3 regions: the 21 amino acid A-

chain and the 30 amino acid B-chain, connected by the C-peptide. It also contains two interchain disulfide bonds between the A- and B-chains, as well as one disulfide bond within the A-chain, prompting the spontaneous folding of the protein molecule. Proinsulin to insulin conversion occurs by cleavage at two dibasic amino acid sequences in the A-/C- chain and B-/C-chain junctions by the action of proteolytic enzymes known as prohormone convertases (PC1 and PC2). This process releases C-peptide, yielding the insulin molecule with the disulfide linked A- and B-chains properly aligned.

Insulin released from the β-cell acts globally in human metabolism in a number of areas, namely: controlling cellular uptake of particular substances, most prominently glucose in muscle and adipose tissue; modifying the activity of numerous enzymes; and, increasing DNA replication and protein synthesis via control of amino acid uptake. Insulin action occurs through binding with the insulin receptor, a receptor with tyrosine kinase activity and with a major intracellular target protein called the insulin receptor substrate (IRS) (15). The effects of insulin on the target cells are both metabolic, particularly promoting glucose uptake, and mitogenic. Metabolic effects are mediated primarily from the IRS proteins through the phosphatidylinositide-3 (PI-3) kinase pathway. These include, but are not limited to increases in glucose transport, glycogenesis in the liver, lipogenesis in adipose tissue, and protein synthesis, as well as corresponding decreases in proteinolysis, lipolysis, and gluconeogenesis (16). Similarly, insulin activation of the PI-3 kinase pathway activates protein kinase B (PKB), stimulating the glucose transport system, leading to the translocation of GLUT-4 glucose transporters to the plasma membrane of peripheral tissues, further increasing glucose uptake. Mitogenic effects are divergently mediated from IRS proteins through Ras and

the MAP kinase pathway. This MAP kinase pathway potentiates but is not critical to the metabolic signaling pathways, nor is the MAP kinase pathway necessary for stimulation of glucose transport.

## 1.2 DIABETES MELLITUS

Diabetes mellitus is the term used to describe a group of diseases marked by high levels of circulating blood glucose resulting from defects in insulin production, insulin action, or both. Diabetes can lead to serious complications and premature death. Diabetes has become a global epidemic, with the World Health Organization (WHO) estimating that in 2000, 2.8% of the world population (7% of North America) had diabetes, expecting the total number of people with the disease to rise from 171 million on 2000 to 366 million in 2030 (17). Of even further concern are estimates that almost one third of these individuals are undiagnosed. Closer to home, the Canadian Diabetes Association has reported that diabetes currently affects over 2 million Canadians, with 60,000 new cases every year (18). The economic costs of diabetes are enormous, with direct medical or indirect expenditures account for approximately one in seven health care dollars spent in North America annually, approximately $9 billion in Canada (19), or $132 billion in the United States (20). These estimates, however, underestimate the true burden of the disease as they omit the intangibles, such as pain and suffering, that diabetes puts on the individuals and families affected by this condition.

### 1.2.1 Etiology & Pathophysiology of Type 1 Diabetes Mellitus

Of all instances of diabetes, approximately 10% fall under the banner of Type 1 diabetes, also known as juvenile-onset or insulin-dependent diabetes. This disease develops as a result of selective immune destruction of the pancreatic β-cells (21) and a subsequent insulin deficiency. While the exact cause of this immune attack is still a matter of debate, it is generally believed that genetic factors play a role in the development of the disease (22;23). Most notable of these are the HLA genes encoded within the major histocompatibility complex (MHC) on chromosome 6p21, designated IDDM1 (24). Nonetheless, studies of twins have shown the disease concordance rate in genetically identical monozygotic twins to be about 50%, versus only 6% among dizygotic twins (25). While this data clearly demonstrates the genetic component of the disease, a less than 100% concordance rate illustrates that genetic susceptibility is not sufficient alone to cause Type 1 diabetes. As such, environmental factors have in the past been proposed to play a role in the development of the disease (26;27), most notable among these being cow's milk (28;29) and viruses (30-32). Clearly, more work needs to be done to determine the relative contribution of each of these genetic and environmental elements.

Type 1 diabetes can lead to a number of serious and potentially life-threatening complications, including heart disease (33), stroke (34), hypertension (35), retinopathy (36), nephropathy (37), and neuropathy (38). Additionally, individuals with diabetes have increased risk of amputations (39), periodontal disease (40), erectile dysfunction (41) and pregnancy complications (42). Fluctuating glycemia levels associated with diabetes and the subsequent accumulation of glucose-based compounds on blood vessel walls are the source of these microvascular and macrovascular effects (43) along with the

all-too-often pre-mature mortality rates in these patients (44). While there is as yet no known way to prevent Type 1 diabetes, a number of treatments have been developed over time both to manage the disease and to attempt to prevent the complications arising from the primary disease.

### 1.2.2 Treatment of Type 1 Diabetes

The general goal of treatment is to improve the glycemic control of diabetic patients (43). So doing prevents the development of potentially lethal diabetic ketoacidosis (DKA) (45), resulting from chronically low insulin levels leading to severe and chronic hyperglycemia (glucose toxicity). Furthermore, improved glycemic control drastically reduces the risk of microvascular complications (eye, kidney, and nerve diseases) (46). Prior to 1922, the only treatment available to diabetics was the strict administration of a near starvation diet, which seemed only to postpone the inevitable death to ketoacidosis (47). This would change in Toronto, Canada in the 1920's.

#### 1.2.2.1 Exogenous insulin

The treatment and care of diabetic patients would be forever changed with the discovery and purification of insulin (8). To this day, daily injections of exogenous insulin have remained the standard of care for patients with Type 1 diabetes and have allowed them to live long and relatively normal lives. Furthermore, findings from the Diabetes Control and Complications Trial have indicated that strict monitoring of blood glucose and intensive insulin therapy can greatly reduce diabetes related complications. This therapy is not without its risks, however. Diabetic patients need daily to perform a sort of juggling act as they attempt to balance diet, exercise, environmental factors, illness, etc.

with their injection regimens, where too little insulin can lead to transient hyperglycemic episodes, and where too much insulin can result in sever hypoglycemia, diabetic coma, and death. Patient compliance to injection regimens is also an issue, particularly among adolescent patients. In more recent years, new insulin formulations have been developed to provide individuals with diabetes an added level of control. Short- and long-acting formulations, and combinations of the two, have provided better maintenance of normal glucose concentration levels, when accompanied with proper planning. Insulin pumps have improved over the years and were developed to provide continuous subcutaneous insulin infusion (CSII) (48). Current models have been shown to reduce the incidences of hypoglycemic and ketoacidotic episodes (49), though they are still not widely used. More advanced "closed-loop" systems involving automatic sensing of glucose and administration of insulin are being investigated, though the technology is still severely limited by the short life of the glucose sensors. Irrespective of the formulation or the method used for administration of exogenous insulin, however, even the best case scenario does not offer the same level of glycemic control as the normally functioning pancreas.

#### 1.2.2.2 Pancreas transplantation

Transplantation of insulin producing tissue has a number of advantages over daily injection of exogenous insulin. To begin with, tissue has the ability to provide a physiological balance by responding to fluctuations in blood glucose concentrations throughout the day to maintain glucose homeostasis, versus injections that require 'best guess' estimates by the patient to determine how much insulin will be needed, and when. Furthermore, subcutaneous injection of insulin diffuses into the blood and travels

systemically, versus the portal circulation that occurs physiologically. As the liver has a role in regulating insulin, transplantation of living tissue into an anatomical site having portal drainage may be ideal (50), though convincing evidence for this in the clinical setting still remains to be seen (51).

Pancreas transplantation was first performed for the treatment of Type 1 diabetes in 1966 (52). While the following 12 years yielded few pancreas transplants due to low rates of graft survival (53), improvements since 1978 in surgical techniques and immunosuppressive drugs, and the establishment of regulated procedures for organ procurement, have led to progressively higher graft and patient survival rates. As of December 31st, 2004, the International Pancreas Transplant Registry (IPTR) has reported more than 23,000 pancreas transplants with one year graft survival rates climbing to 85% for simultaneous pancreas-kidney cases, 78% for pancreas after kidney cases, and 77% for pancreas transplant alone cases, with patient survival exceeding 95% in all categories (54). These transplants have been shown to provide a state of stable normoglycemia to recipients (55) and may provide benefit and some restoration of function to patients with nephropathy (56), neuropathy (57), retinopathy (58), and macrovascular disease (59). Despite these benefits, the extreme invasiveness and complicated nature of the surgery, along with the lifelong need for immunosuppression, limits this therapy to only the most severe diabetics and those requiring renal transplant (60).

## 1.3 ISLET TRANSPLANTATION

In addition to the aforementioned complexities of pancreas transplantation, there also remains the fact that the procedure involves the transplantation of far more tissue than

what is actually necessary. As the islets only comprise approximately 2% of the pancreatic mass (61), and given that the presence of exocrine secretions in pancreas transplantation can lead to their own problems such as sepsis and urological complications (62;63), it would seem logical to attempt to pursue techniques allowing the transplantation of pancreatic endocrine tissue alone.

While initial attempts at islet isolation were largely unsuccessful (64), isolations using collagenase enzyme digestion of pancreatic fragments to free islets from the surrounding exocrine tissue greatly improved the process (65). Further refinements increasing islet yield were made by Paul Lacy, who developed a method involving intraductal distention and digestion of the pancreas with collagenase, mechanical disruption of the islets from the pancreas, and density centrifugation to separate islets from exocrine remnants (66). These processes lead to the first successful transplantation of intact rat islets by Ballinger and Lacy in 1972 (67). Further advancements were made later when ficoll was substituted for sucrose on density gradients (68), ficoll providing a better osmotic environment for the islets.

Early animal experiments in islet transplantation proved to be highly successful, at least in rodent models, with transplanted islets sustaining demonstrable insulin secretion (66;67), and even a biphasic insulin secretory response to glucose (69). Despite these successes, it became apparent that the techniques involved in the isolation of islets from small animals did not translate well into larger animals such as the dog (70) or human (71), due in large measure to the size and more fibrous nature of the pancreas. Despite the struggles, advances continued to be made by a number of groups that continued to have faith that this therapy could have clinical relevance in the future.

The first human islet transplants were performed in the 1970's (72), though normalization of blood glucose and insulin independence was only rarely reported (73). As with the animal trials, several advances in human islet transplantation were made by investigators over the course of the following two decades. In particular, the development of the Ricordi digestion chamber (74) and the development of a protocol for the controlled perfusion and distension of the pancreas with the digestive enzyme collagenase (75;76) greatly improved islet purity and yields. Nontheless, when the Islet Transplant Registry reported in 1992 the results of the 90 procedures between 1974 and 1989, only 5 patients (5.6%) were insulin independent after one year (77). Improvements were made during the following decade – such as the introduction of the COBE continuous purification system (78) and the use of purified enzyme blends with low endotoxin content (79) – when 237 procedures were performed between 1990 and 1999, though still only 27 of the patients (11.4%) remained insulin independent after one year (80). It seemed clear that some change needed to be made in the existing protocols of the time for islet transplantation to provide for diabetic patients a real hope of living a life without insulin.

### 1.3.1 The Edmonton protocol

In 2000, the culmination of thirty years of work led the group at Edmonton to announce in a landmark paper that they had managed to normalize blood glucose by islet transplantation without exogenous insulin in 100% of 7 patients for greater than 1 year (81). This new protocol in particular involved improvements with respect to an increased mass of transplanted islets and a new glucocorticoid-free immunosuppression regime. The announcement led to the establishment of multi-center clinical trials with subsequent

success in other centers (82), with success rates varying between centers due to site specific modifications and improvements in the experimental protocol (83-86). In fact, success rates were so dramatically improved that from the announcement in 2000 to mid 2005, a total of 471 patients with Type 1 diabetes in 43 institutions worldwide had received islet transplant therapy (87), more than had received islet transplants in the entire 30-year history preceding the Edmonton protocol. One year follow up rates continue to be impressive in most established centers. In a total of 118 patients in Edmonton, Minnesota and Miami, 82% were insulin free at 1 year (83). In another study of 36 patients at nine international sites, 80% of recipients at the three most experienced sites was over 80% (82).

Though early results have been promising, it is clear also that the long term efficacy of this islet transplant therapy remains somewhat uncertain. In a follow-up study of patients transplanted at Edmonton it was found that only a minority of patients (approximately 10%) remained insulin independent at five years, though about 80% seemingly retained some level of graft function (88). Furthermore, a number of patients developed complications as a result of immunosuppressive therapy, such as mouth ulcers, edema, anemia, diarrhea, weigh loss or ovarian cysts. Still others developed hypertension, pneumonia or macroproteinuria. Three patients had pneumonia, two seroconverted to CMV and many developed an increased need for lipid-lowering medications. One patient developed papillary carcinoma of the thyroid. Despite the advances, there are clearly deficiencies in islet transplant therapy yet to be overcome in order to make this treatment a stable, long-term solution to the problem and complications of Type 1 diabetes.

### 1.3.2 Limitations of islet transplantation

Though much less invasive than pancreas transplantation, islet transplant surgery is not free from complications. In the five-year follow-up from Edmonton, a number of acute complications were associated with the procedure (88), namely: portal vein thrombosis (n=5), punctured gall bladder (n=2), elevated average portal pressure and elevated average liver transaminases. Each of these complications, though not without risk, were easily managed or resolved with time. Of larger concern were complications associated with the immunosuppression regime of tacrolimus, sirolimus, and daclizumab necessary to prevent immediate rejection and destruction of the islet graft (81). Immunosuppressive drugs are not well tolerated by all patients, the benefits of which need to be weighed against the potential risks and complications, mentioned earlier. Furthermore, the whole area of islet transplantation suffers from a mammoth shortage of donor tissue, evidenced in the mere hundreds of procedures done in the face of hundreds of millions of diabetics world wide. We shall hereafter focus our attention on the challenges faced by investigators in field of islet transplantation to two main areas: the shortage of islet supply, and the problem of tissue rejection.

#### 1.3.2.1 Source/supply

Recent data obtained from the Edmonton group has reported that and average of nearly 800,000 islets were required among recipients to achieve insulin independence (88). Compound this finding with the multiple pancreas donors needed to supply this number of islets to each recipient, with the variations in islet isolation yield between each pancreas (for not every pancreas obtained makes it to transplant) (89), and with the

overall shortage of organ donors, we can see that we just need more islets. Even highest-yield preparations only recover 20-50% of the potential islet mass (90), and though increased numbers of islets per pancreas may obtained through improvements in pancreas procurement (91-93) and islet isolation (94-96), it is not enough. The ability to use single versus multiple donor transplantation to achieve insulin independence (84) could potentially double the number of graft recipients; however it is not enough. The use of non-heart beating donors has been proposed as a means of increasing the pool of available pancreata (85), but it is not enough. Also, the use of living related donors to provide pancreatic segments (97;98) for islet isolation has recently been considered as a potential source of transplant table islets. Still, this does not provide enough tissue to manage the millions of patients with Type 1 diabetes. Clearly, if ever investigators are to manage Type 1 diabetes independent of daily insulin injections, alternative reserves of transplantable tissue must be found. We shall explore these potential sources of shortly hereafter.

#### 1.3.2.2 Immune rejection

It has already heretofore been discussed the complications resulting from the immunosuppressive drugs used to prevent rejection of islet grafts. Nonetheless, were it not for these medications the engrafted islets would have no chance of survival beyond one week, much less one year, as allograft rejection is an immunologic inevitability. It has also been shown that islets exposed to ABO-compatible blood triggers a blood-mediated inflammatory reaction instantaneously, leading to clot formation, leukocyte infiltration and subsequent islet damage (99;100). Furthermore, the primary pathology of Type 1 diabetes, namely the autoimmune attack of pancreatic β-cells, may unless treated

lead to the selective destruction of transplanted β-cells, just as in the native pancreas (101). In order for islet transplantation to be reasonably applicable to all Type 1 diabetic patients, researchers and clinicians must develop immunosuppressive regimens that minimize the side effects of such therapies, or alternatively develop strategies that overcome the need for chronic systemic immunosuppression altogether. Much of the focus of our present work has been focused on exploiting such strategies, and as such, alternatives to immune suppression shall be discussed later in this text.

## 1.4 ALTERNATIVE SOURCES OF TRANSPLANTABLE ISLETS

Due to the extreme shortage of human cadaveric islets, it is essential to find an alternate source of transplantable tissue is this treatment is to become widespread therapy among the population of diabetic patients at large. This tissue would need to adhere to a number of criteria in order to be a suitable replacement for the allogeneic islets currently used in clinical trials. First, and obviously, the tissue would need to produce insulin. This could potentially occur naturally, as in islets, or could be the result of manipulating non-islet cells to produce insulin. Furthermore, this tissue would need to produce enough insulin to warrant the procedure; that is, they would need to produce enough insulin to normalize blood glucose, or at least reduce the requirement for injection. Next, the transplanted cells should respond to physiological stimuli in a manner similar to pancreatic β-cells, particularly in response to changes in blood glucose concentrations. We would want to ensure that insulin is released in response to a meal, for example, but not so much insulin that the transplant recipient becomes severely hypoglycemic. Ideally, the transplanted cells would maintain stable normoglycemia just like native β-cells. Finally, we would hope that transplanted cells would be as non-immunogenic as possible. This could

potentially reduce or eliminate the requirement for immunosuppressive medications, along with their associated side effects and complications.

### 1.4.1 Cellular approaches

A number of cell-based approaches have the potential to provide an abundant supply of insulin producing cells. One potential approach is through the establishment of an immortalized β-cell line (102). Such lines have been reported to produce near physiological amounts of insulin (102;103) and have been shown to normalize blood glucose concentrations in mice (104;105). Another cell based approach is the use genetically modified of non-β-cell lines – cells that do not naturally produce insulin but are bioengineered to produce and release insulin via gene transfection. Such cells that could be engineered to produce insulin might include K-cells from the gut (106), myoblasts (107), fibroblasts (107), and hepatocytes (108;109). While it seems as though there may be a whole myriad of cells that might potentially be used in place of pancreatic β-cells, these various cell lines are not without their shortcomings. To begin with, some of these cell lines have been shown to de-differentiate in culture, subsequently leading to changes in insulin release dynamics (103;104). Also, it can be difficult to replicate all of the molecular machinery necessary for glucose sensing, insulin production and insulin release in cells not naturally predisposed to do so. Finally, some of these cell lines are often shown to have unregulated proliferation potential *in vivo* (102), subsequently leading to insulin overproduction, severe hypoglycemia and death of the recipient. What is certain is that much more work needs to be done before any such cell lines can be used in the clinical realm.

### 1.4.2 Islet progenitor cells

Other cells need not be unnaturally manipulated to grow or to produce insulin – the potential to do so lies within the cell itself. These cells may exist as stem cells or precursor cells within the pancreas itself and could potentially be differentiated to become actual islets. A number of investigators have published data supporting the thought that the ductal cells of the pancreas have the ability to act as pancreatic precursor cells (110;111). Others have suspected that it is rather a specific sub-population of cells within the pancreatic ducts that are the islet precursors (112;113). Others have hypothesized that these precursor exist within the islet itself (114;115). Still others hypothesize that islets may develop from a dedifferentiation of exocrine cell of the pancreas into ductal cells and subsequent re-differentiation event into endocrine cells (116-118). Extensive study has been done trying to determine the events and factors involved in islet neogenesis, and while much uncertainty exists, what is certain is that the endocrine pancreas is a dynamic organ, responding to growth, development, and changes in conditions. As more is understood about the dynamic pancreas, the potential exists to greatly expand the mass of donor islets through the expansion of cadaveric donor islet precursors. Furthermore, the potential exists also to activate β-cell neogenesis in the pancreatic tissue of the Type 1 diabetic patient, either *in vivo* or *ex vivo*, allowing regeneration of the patients own insulin producing tissue and eliminating the need for chronic systemic immunosuppression in that patient.

### 1.4.3 Embryonic stem cells for islets

Human embryonic stem (ES) cells are derived from the inner layer of the blastocyst (119;120). The wide interest of these cells in the field of medical research lies in the ability of these cells to self renew and differentiate (121;122), theoretically giving these cells the ability to differentiate into any definitive cell type when exposed to the proper signals over time. Furthermore, with the establishment of ES cell lines it could be possible to develop an unlimited supply of cells for transplantation. Thus, the hope is that study will lead researchers to discover the necessary cues to transform these cells into glucose-sensitive insulin producing endocrine β-cells (123). Already, some investigators have demonstrated the ability to obtain insulin-producing cell clusters from human ES cells (124;125). Furthermore, it has been shown in animal models that ES cells differentiated into islet-like structures have the ability to correct hyperglycemia when transplanted into diabetic animals (126;127). Even more promising is the proposal that ES cells may have reduced immunogenicity (128), as in the undifferentiated state they have been proposed to be immunologically undefined and programmed by the host's own immune system as "self" tissue (129). Furthermore, these cells could also be manipulated prior to transplantation to induce immune-compatibility with the intended recipient (128;130). Unfortunately, the use of ES cells in the clinical realm is not without its obstacles to overcome. Like other cell lines, much work yet needs to be done to determine the factors and timing necessary to transform an undifferentiated stem cell into something resembling a β-cell. Also, understanding mechanisms of cell expansion, particularly how to turn it on and off, is key before moving into the clinic in order to avoid teratoma formation in transplant recipients (119). Finally, there are the various

ethical and religious sensitivities concerning the use of human embryonic tissue for medical research (131;132), which makes the future of embryonic stem cell research somewhat uncertain.

### 1.4.4 Xenografts

With the shortage of tissues of human origin, it becomes prudent to look to other potential sources for transplantable tissues. Xenotransplantation refers to the use of tissues from non-human species for transplantation into humans. Xenotransplantation is not a new concept and has been studied and even used for years (133), particularly in the realm of heart and pulmonary transplantation (134). Even in the treatment of diabetes, xenotransplantation was first considered over a century ago when Watson attempted to cure diabetes in a 13-year-old child through subcutaneous implantation of sheep pancreas fragments (135). A number of animal models have been studied in an attempt to find suitable donors, and while much has been learned, a number of obstacles still need to be considered to make xenotransplantation feasible.

#### 1.4.4.1 Potential sources of xenogeneic tissue

A number of species have been considered as potential sources of tissues for islet xenotransplantation. Whereas non-human primates (NHP) may initially seem to be the most obvious choice given their genetic and anatomical similarities to humans, there are a number of problems associated with using NHP's as donors. These animals do not breed well in captivity and have relatively few young over the course of their lifetime compared to other large mammals. Also of concern is the risk of cross-species disease transmission (136), given the concordance between these species and the similarity between NHP and

human pathogens. As such, other discordant species have been considered as islet donors, more notably the cow (137;138), the pig (139;140), and the islet-like structures known as Brockmann bodies of teleost fish (141;142). As the consensus among many investigators regards the pig as the species of choice with regards to xenotransplantation (143), we shall focus our discussion hereafter on the use of porcine islet transplantation as a treatment for Type 1 diabetes.

#### 1.4.4.2 Pig

Pigs have a number of characteristics that make them very appealing for xenotransplantation donors over NHP's or other animals (144). Pigs breed rapidly and have large litters. Pigs are domesticated and can be easily housed in a specific pathogen-free environment. Though an immunologically discordant species to humans, they are anatomically, physiologically and biochemically similar to humans and have shown some compatibility with humans (143). As an example, porcine insulin differs from human insulin by only one amino acid (145). Furthermore, pigs have the added advantage of being quite easily genetically manipulated, potentially addressing some of the rejection issues surrounding xenotransplantation (146). Results were poor, however, in attempts to isolate of adult pig islets (147), due largely to the inability to obtain a relatively pure islet preparation (148) and the overall fragility of the islets (147;149). Fetal porcine islets were isolated from the pancreas with greater success, though given the immaturity of these islets, they tended to exhibit poor insulin responsiveness to glucose (150;151). What was needed was a means of obtaining best of both adult and neonatal islets: islets that were easy to isolate, produced sufficient yields, and which showed acceptable insulin responsiveness to a glucose challenge.

#### 1.4.4.2.1 Neonatal porcine islets

In the early 1980's it was shown that islets isolated from neonatal (newborn) pigs were capable of secreting significant quantities of insulin in response to a glucose challenge *in vitro* (152;153). Then, in 1996, Korbutt *et al.* published a method for the large scale isolation of neonatal porcine islets (NPI) (154). Furthermore, results published that these islets survived well in tissue culture, during which time the total β-cell mass increased 150%. These islets had the ability to correct hyperglycemia in 100% of diabetic mice receiving 2000 islets, and when recovered at 14-weeks post-transplantation had a 20- to 30-fold increase in cellular insulin than at the time of transplantation. Subsequent experiments have shown evidence that suggests that these relatively immature islets have the ability to grow and mature both in tissue culture (139) and in vivo (155). Further experiments in Edmonton and elsewhere have also shown the ability of these NPI's to restore normoglycemia diabetic animals both small (154;156) and large (157;158).

What is most exciting about advances in the use of neonatal pig islets is their potential to move quickly into clinical trials. It was shown in Sweden in the early 1990's (140) and subsequent follow-up studies (159-161) that fetal porcine islets transplanted into human recipients failed to demonstrate any adverse effects to recipient patients. Furthermore, though none of the recipients became insulin independent, four patients in this trial demonstrated signs of xenografts function (C-peptide release) for 200 to 400 days post-transplantation. Given the immature nature of fetal islets versus the incredible growth, differentiation and insulin release potential of neonatal porcine islets, it would seem hopeful that NPI's would be effectual in providing a measure of insulin-relief to primates and humans. In fact, a recent study in *Nature Medicine* demonstrated the ability

of transplanted NPI's to restore insulin independence to rhesus monkeys with only minimal immune suppression for up to >260 days (162). This announcement signifies a huge leap forward in islet transplant research, and is one major step closer to advancing neonatal porcine islet transplantation into the clinical arena. Furthermore, considering the fact that a single litter of pigs could potentially produce enough islets to transplant two human diabetics (163), this therapy has the added advantage over any other available modality of being able to treat a significant proportion of the population of diabetic patients at large.

#### 1.4.4.3 Barriers to xenotransplantation

The obstructions to clinical xenotransplantation fall under three broad headings. First, there are a number of ethical issues surrounding xenotransplantation (164). Formation of the Ethics Committee of the International Xenotransplantation Association (165) and other such organizations are the result of such issues. While a discussion on this matter is of a scope far larger than the constraints of this work, it is nonetheless of relevance to add that there will inevitably be persons of various nations or religious convictions for whom xenotransplantation will not be an option. Next, the risk of cross-species infection or disease transmission (xenozoonosis) requires significant attention to ensure that the donor tissue is in fact safe to be transplanted into human recipients. It would be irresponsible to transplant a patient with tissue that may not only instigate disease in the recipient, but in the case of endogenous retroviruses potentially to their progeny as well. Finally, to become a clinical reality we must ensure that the effects of host immunity are addressed in order to ensure the success of the transplanted xenogeneic tissue. Potentially, this could be the result of either treating the donor of interest, or the recipient, or both.

#### 1.4.4.3.1 Xenozoonosis

Certainly, in an age when SARS and Avian flu make general public fearful of disease transmission from animals, the issue of xenozoonoisis from xenotransplantation must be approached with great prudence. Much of the attention has focused on the pig with regards to xenograft disease transmission to humans. Within the genome of the pigs are found porcine endogenous retroviruses (PERVs), and a great deal of concern has focused on the potential risk of this retrovirus replicating and initiating disease in human recipients or their families. While there has been no indication as yet of PERV transmission to humans working with (166) or receiving transplants of porcine tissues (160;161;167), nor has it been shown that PERV is capable of a productive infection (168;169), it has nonetheless been shown that there is a risk of PERV migrating from implanted tissue into the blood (170) and peripheral tissues (171) of the recipient, and that PERV can in fact infect human cells (172). A recent study also noted that PERV expression is up-regulated briefly after transplantation (173). These concerns are further amplified by discoveries that these retrovirally infected cells may be able to evade immune destruction (172) and that these cells may potentially give rise to retrovirally induced cancers such as leukemia (172;174). New studies indicate that the treatment of the PERV problem may be possible through the use of RNA interference (175;176), and naturally occurring human antibodies have been shown to prevent transmission of PERV to human cells (177;178). Still, further study on PERV is needed, and continued long-term follow-up of recipients of porcine tissue is certainly warranted, not only to determine the risk of infection, but particularly to determine if PERV is pathogenic to humans.

#### 1.4.4.3.2 Xenograft rejection

Multiple immunological barriers must be overcome if pig-to-human transplantation is to become successful, though of greatest consequence among the various xenoreactive antigens are the presence of glycoproteins containing the terminal carbohydrate galactose α(1,3)-galactose (α1,3-Gal). This epitope is generated by the enzyme α1,3-galactosyl-transferase (α1,3-GT), which is absent in humans, apes and Old World monkeys, though it is present in the vascular epithelium of other mammals. α1,3-Gal is also a cell wall component of gastrointestinal bacteria, and as such, anti-α1,3-Gal antibodies develop early in life. Binding of these naturally occurring xenoreactive antibodies (XNA) in human sera to α1,3-Gal antigens present xenograft cells initiates an immune process known as hyperacute rejection (HAR). Antibody binding activates compliment, rapidly destroying the graft within 2 days through an intense inflammatory response (179). Acute humoral and cellular rejection may also develop subsequent to HAR (167). Innate and adaptive responses to xenografts must not be underestimated [reviewed in (180)]. Finally, as Type 1 diabetes is an autoimmune disease, the recurrence of the autoimmune disease and subsequent destruction of the transplanted xenograft by the original diabetogenic immune response must be considered (101). It is not totally certain whether xenogeneic islets can be recognized by the specific autoimmune repertoire, though there is some evidence that this may be of little concern, as the effectors of autoreactivity may be species specific (138). Nonetheless, it is certain that graft rejection is a major obstacle to the success of xenotransplantation, the treatment of which warrants intense study and review.

## 1.5 CIRCUMVENTING REJECTION OF TRANSPLANTED ISLETS

In addition to protecting the well-being of the recipient, investigators in the area of islet xenotransplantation aim to secure the safety and longevity of the donor graft within the recipient. In attempting to meet both these goals, one or more strategies may be employed. First, therapies can be employed to reduce host immunity prior to and/or following transplantation of xenogeneic tissues. Next, donor animals can be addressed, in an attempt to find or develop animals of reduced immunogenicity. Finally, donor tissue can be treated following recovery from the donor, but prior to transplantation in an attempt to make it more immune-compatible with its potential host. The remainder of this treatise shall be concerned with a review of techniques for protecting islet grafts from host immunity.

### 1.5.1 Reducing recipient immune responsiveness

Methods for reducing the ability of the recipients to form an immune response to an islet transplant fall into three areas. To begin with, the use of immunosuppressive medications can be used to ameliorate graft survival by reducing the ability of immune effector cells to mount a response to the implanted cells. Next, other techniques have been studied that attempt to hide transplanted cells by tolerizing the host immune system to the graft, making the graft appear as though it were 'self' tissue. Finally, within the body there exist specific sites that are in themselves immune compromised and which provide a level of protection to both foreign and self cells existing within the localized environment. Each of these techniques shall briefly be discussed.

#### 1.5.1.1 Immunosuppression

As mentioned earlier, current clinical islet transplantation protocols involve the use of three primary immunosuppressants, sirolimus, low-dose tacrolimus and daclizumab, along with oral antibiotic prophylaxis to prevent post-surgical infection (81). These primary medications prevent rejection of the implanted islet by systemically suppressing the immune function of the recipient. While this does slow the rate of graft failure, it also results in secondary complications and side effects (88). As a result, this therapy may be poorly tolerated in many patients. Systemic suppression of immunity opens the host up to a myriad of potential infections, parasites and cancers that might otherwise be easily handled by regular immune function. New approaches have sought to more specifically inhibit host immunity to xenogeneic cells through the use of monoclonal antibodies (mAb). As an example, CD154 mAb has been used to specifically bind and deplete circulating anti-Gal antibodies in recipients with encouraging results (181;182). Combinations of multiple mAb's have more recently been used as a means of adding even further specificity to the therapy (183;184). Similar to mAb therapy, infusions of specific synthetic Gal oligosaccharides into graft recipients have been used to competitively bind and deplete anti-Gal antibodies (185;186). Such therapies provide specificity of response and reduce the susceptibility of human antibody/complement cytolysis of the graft in the recipient while minimizing potential side effects, making such treatments appealing for continued study and future therapeutic use.

#### 1.5.1.2 Induction of immune tolerance

Ultimately the hope is that islet transplantation will be able to restore normal glucose responsiveness in the absence of exogenous immunosuppressive therapy. Tolerance defined is a permanent state of immune unresponsiveness of the recipient to the islet graft. The ability to induce such a state would allow clinicians the opportunity to extend this therapy to recipients for whom islet transplantation was not previously possible. A number of experimental procedures to induce tolerance are currently underway and have shown promise. One approach includes the selective withdrawal or 'weaning' of patients from immunosuppressive medications or monoclonal antibody therapies (187-189). Another such strategy to induce tolerance involves the co-stimulatory blockade of T-cells, effectively preventing T-cell activation and clonal expansion. Of particular interest here are the monoclonal antibodies CTLA immunoglobulin (190) and anti-CD154 (191;192) (anti-CD154 you may recall also has an anti-Gal antibody depleting effect, and is thus particularly effective). Similarly, the use of potent T-cell depleting agents at the time of transplantation has been shown to be an effective strategy for facilitating tolerance and long term survival to islet xenografts (193;194). Myeloablation followed by bone marrow transplantation has been shown to induce mixed hematopoetic chimerism and has proved to be perhaps the most successful means of inducing tolerance in the clinic (195;196). Clearly, tolerance induction is an exciting area of study, and though it has only been reviewed briefly here, more thorough reviews of the topic are available (180;197).

#### 1.5.1.3 Transplantation to immune compromised sites

A form of tolerance exists whereby foreign tissue can be implanted into one of a number of naturally occurring sites in the body in the absence of immune suppression with little or reduced risk of rejection (198). Some of these locations include the testes (199), brain (200) and thymus (201). Furthermore, islets implanted into each of these sites have shown improved or indefinite survival compared with non-privileged sites. The mechanism of this protection certainly includes the blood tissue barriers (202;203), but it also extends beyond these physical barriers to include an immune system actively involved in this protection (203). Immune privilege in these sites likely also involves the induction of apoptosis in invading T-cells (204) and mononuclear cells (205) as a means of prolonging the survival of foreign cells transplanted into these sites. This reaction is mediated by, but not restricted to the interaction of Fas ligand receptors in these sites with Fas receptors on invading immune cells (205). Unfortunately, islet transplantation into these sites is limited clinically by a number of anatomical constraints, such as the size of the site, or in the case of the testes, the complete absence of the site in roughly half the population. As such, investigation of these sites has largely focused on the mechanisms of protection provided by specific effector cells. Of particular interest are the Sertoli cells of the testes.

##### 1.5.1.3.1 Sertoli cells

Sertoli cells are found within the seminiferous tubules of the testes where they function to nurse and support developing germ cells (206). Sertoli cells have also been shown to form tight junctions creating a blood-testes barrier, providing a site of immune-privilege

to germ cells inside the barrier (207;208). In addition to providing this barrier, Sertoli cells have a role in the prevention of immune rejection of host germ cells through the secretion of numerous factors that have been shown to have a role in local immune tolerance (209-214). Sertoli cells have particularly developed the interest of researchers for their ability to be isolated from and transplanted distally to the testes to form a surgically created site of immune privilege (215;216). Sertoli cells have been shown to secrete a number of factors, many of which are involved in active immune suppression. These include proteins involved in transport and bio-protection; proteases and protease inhibitors; basement membrane proteins; hormones and growth factors; and many other factors involved in immune regulation. While a thorough review of these regulators is too extensive for the purposes of this work, they are nonetheless reviewed in greater detail elsewhere (217;218). What is of importance for our purposes is that, taken together, Sertoli cells transplanted into a foreign site have been shown to provide an immunoprotective (204;209), anti-inflammatory (219;220), and tolerizing (221;222) environment in the immediate vicinity of the Sertoli cell graft.

#### 1.5.1.3.1.1 Co-transplantation with islets

As a result of this locally created environment of immune privilege, Sertoli cells have been of great interest o researchers in the area of islet transplantation. As a result of the fact that both islets and Sertoli cells are transplanted as cellular grafts, these cells can be transplanted together in the hopes of the Sertoli cells creating a microenvironment of protection for the co-engrafted islets. Furthermore, in the case of neonatal porcine islets, it is hoped that the 'nurse cell' properties of Sertoli cells will provide factors for the aided growth and maturation of the islets. Co-transplantation studies have clearly shown that

Sertoli cells have some ability to protect pancreatic islets from allograft (215;216) and xenograft (223;224) rejection. Additionally, there is evidence that Sertoli cells have some ability to protect β-cells from a recurrence in autoimmune destruction (210;211). This model for providing an environment of tolerance for transplanted islets shows great promise clinically, may provide an excellent means of delivery for xenogeneic islets into humans, and is currently under investigation clinically (225).

### 1.5.2 Reduction of donor animal immunogenicity

Alteration of donor animals for xenotransplantation is an appealing prospect for reducing the immunogenicity of porcine donor tissues. In particular, the creation of transgenic pigs has evolved as a popular prospect. Genetically engineered galactosyl-transferase gene "knockout" pigs are produced by inactivating the α1,3-GT gene in an attempt to evade hyperacute rejection (226). RNA interference can be used similarly to downregulate α1,3-GT expression to decrease porcine endothelial cell immunogenicity (227). Other transgenic pigs have been developed expressing human complement inhibitors to surmount immune destruction of the xenografts (228-230). Genetically manipulated animals are not without their shortcomings, however. Unfortunately, in eliminating HAR, PERV derived from infection α1,3-GT knockout pigs may have increased infectious potential (231). Furthermore, when hyperacute rejection can be prevented, acute humoral and cellular rejection develops (167). While the development of immunologically compatible xenogeneic donors is ideal, further refinement and understanding is necessary to achieve greater success.

### 1.5.3 Pre-transplantation treatment of donor islets

In addition to developing genetically compatible transgenic animals, transplantable islets can be treated prior to transplantation in an attempt to reduce their ability to elicit an immune response or be destroyed by such a response. In fact, one the of the great tenets of islet transplantation, and in fact cellular transplantation in general over whole organ transplantation is the ability to work with the cells of interest prior to their transplantation and function in recipients. For example, there is evidence that the immunogenicity of islets may be reduced by tissue culture (232), antibody treatment (233), UV irradiation of the islets (234) or cryopreservation (235;236) of islets prior to transplantation. Improving islet isolation techniques in order to increase the purity of islet preps could arguably decrease the immunogenicity of the graft be reducing the vascular content and subsequent α1,3-Gal content of the graft (237). The reduction of α1,3-Gal through enzymatic cleavage of α1,3-Gal or its replacement with some innocuous oligosaccharide has also shown potential in rendering pig cells less immunogenic to the human immune system (238;239). Furthermore, islets transfected with genes protecting against apoptosis (240) or genes inducing islets to produce their own immunoprotective cytokines (241) prior to transplantation could potentially protect islets and greatly improve graft survival.

#### 1.5.3.1 Immune barriers

In addition to specifically treating the cells within the islet to reduce donor immunogenicity, there exists another strategy for the pre-transplant handling of the islets. Whereas the use of immunosuppression, the creation of transgenic animals, and the manipulation of islet cells prior to transplant involves the creation of a conceptual barrier

around the islet to prevent immune destruction of the graft, the next approach involves the creation of actual physical barriers around the transplanted tissues for protection. Thus, these technologies may act in principle much as the naturally occurring sites of immune privilege in the body, though they are artificially created. This technology involved devices that could be placed in a number of different sites within the body and may have either direct vascular access or may operate by diffusion of nutrients and products into the interstitial tissues. Intravascular devices allow the flow of blood through the lumen of some sort of porous tube that houses the islets, whereas extravascular devices function by diffusion and do not require vascular anastomoses (reducing the risk of thrombosis). Furthermore, islets can be placed in devices where all of the implanted cells are housed within the same chamber (macroencapsulation), or islets can be individually enveloped in their own membrane (microencapsulation). Regardless of the means of encapsulation or location of the implant, these devices must allow the selective permeation of glucose, nutrients and oxygen in and the passage of metabolic wastes and secreted factors such as insulin out, all while protecting from host immunity. Furthermore, the encapsulation material itself should be immunologically inert.

#### 1.5.3.1.1 Immunoisolation devices

The use of a semi-permeable membrane for protection of islets from immune attack was first published by Chick *et al.* in 1977 (242). Since then, researchers and industry working in this field have evolved into the study of two types of macroencapsulation device: vascular biohybrid devices and diffusion chambers. Vascularized bioartificial pancreas (VBAP) devices (243) consisted of a single-coiled acrylic polymer in a disc

shaped acrylic housing could be anastomosed into the vascular system. These devices allowed the exchange of products between the blood and luminal space of the device, and showed some promising results, particularly in dogs (244). Unfortunately, the period of insulin independence was only brief, and thrombosis associated with the presence of the islets was a major problem and cause of failure (245). Hollow fiber (246;247) and flat disk-shaped (248;249) diffusion chambers were also investigated. Experiments explored the use of a variety of materials of differing porosities in an attempt to improve the immunoprotective and biocompatibility properties of these devices, with varying success (reviewed in (250)). One of these diffusion chambers, the TheraCyte device (Baxter Healthcare, Round Lake, IL, USA) even had the added advantage of a double layer polyester-Biopore membrane (248) that could be subcutaneously implanted and neovascularized prior to islet transplantation into the device (251). Unfortunately all these devices were severely limited by lack of nutrient availability caused by the high density of islets within the device and the extensive fibrosis that tended to develop on the surface of these chambers over time.

#### 1.5.3.1.2 Microencapsulation

As an alternative to larger immunoisolation devices designed to contain hundreds or thousands of islets in one central chamber, microencapsulation employs the use of stable, purified biocompatible materials to surround and protect individual islets from immune destruction. Microcapsules offer a number of advantages over other encapsulation devices, including greater surface area to volume ratio, ease of implantation, and retrievability by lavage and aspiration, if necessary. While the mechanisms by which microcapsules protect islet xenografts from host destruction are not fully understood, it

has been suggested that prevention of cell-cell contact with host lymphocytes may be a factor (252). Another advantage is the ability to manipulate these microcapsules for their biocompatibility (253;254), size (255;256), loading density (number of islets per capsule) (257), porosity (258;259) and rate of degradation (260), all of which have effects on the subsequent survival of the islets. A number of materials have been used in the past for encapsulation of islets. Among these are a number of synthetic materials, such as isopropylacrylamide (261), hydroxyethyl methacrylate-methyl methacrylate (HEMA-MMA) (262), polyethylene glycol (PEG) (263), and polyacrylonitrile (AN69) (264). Also, a number of naturally occurring biomaterials have been used for encapsulation of islets, including cellulose (265), agarose (266), chitosan (267) and alginate (268). Furthermore, variations of capsules combining these various substrates, along with other materials, have been explored (269;270). Despite the various materials used, however, alginate has predominated as the material of choice in the field of islet encapsulation as it has been shown to remain stable for up to years in small and large animals and found to not interfere with the cellular function of the islet (271). As such, alginate encapsulation of islets shall be the focus of the remainder of our discussion.

#### 1.5.3.1.2.1 Alginate microcapsules

Alginate was first used in 1977 by Kiestan and Bucke to study the effects of alginate immobilization on microbial cells. Then, in 1980, Lim and Sun published results indicating that islets immobilized in alginate gels could be transplanted into diabetic rats and correct the diabetic state for 2-3 weeks (268). This paper subsequently inspired more than 25 years of research among investigators attempting to optimize this procedure and extend the period of normoglycemia in transplanted animals indefinitely. To better

understand this technology, we will discuss the make-up of alginate, some of the past successes and challenges in this area, and discuss potential future directions for success.

#### 1.5.3.1.2.1.1 Composition and properties of alginate

Alginate, a derivative of brown algae, is a linear polysaccharide consisting of 1,4-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G). Monomers can appear in chains of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks), alternating M and G-residues (MG-blocks), or in randomly organized blocks. Chemical composition, primary structure and average block lengths are determined by NMR spectroscopy (272). The relative amount of each block type varies with the geographical origin of the algae from which it is obtained as well as the method used for the alginate extraction (273). The physical-chemical and biological properties of alginate vary widely with this differing composition. Alginate gels instantaneously in the presence of divalent cations like $Ca^{2+}$ or $Ba^{2+}$ and occurs when the divalent cations take part in the interchain ionic binding between guluronic acid blocks (G-blocks) in the polymer chain, giving rise to a three dimensional network. Consequently alginates with a high content of G-blocks induce stronger gels with a more stable and more porous network. Gels made of M-rich alginate are softer, more fragile and have a lower porosity due to the lower binding strength between the polymer chains and to the greater flexibility of the molecules.

A number of issues require attention with respect to using alginate as a material for encapsulation. Biocompatibility is a significant issue when working with alginate, or any materials used in transplantation for that matter. Alginate biocompatibility is dependent on a number of factors. Foremost among these is the relative purity and compositions of the alginate (274). Crude forms of alginate are known to contain a

number of impurities known to provoke inflammation (275-277), and the careful purification of the alginate has been shown to considerably reduce its inflammatory potential (254) and demonstrate the need for using pure alginate preparations (278). Furthermore, the biocompatibility of the alginate may be influenced by the relative M- and G-contents of the alginate (276-278), though there still remains some discussion as to whether high G or high M alginates are more ideal. Also, biocompatibility is a function of the M- and G-contents of the alginate which affects whether islets are adequately encapsulated (279) and the relative smoothness of the capsule surface (271). Finally, size of the capsules generated is of great interest. Smaller capsules have the advantage of a higher surface to volume ratio, allowing good transport of essential nutrients. Smaller capsules are also less fragile. Diffusion limitations with larger beads may limit cellular metabolism, as the lack of oxygen and essential nutrients to the interior of the capsule may lead to death of the islet to starvation (280). In overcoming these issues, great refinements have been made in the purification and production of alginate microcapsules (281-283), which has led to increasing promise and success *in vivo* with this transplantation technology.

#### 1.5.3.1.2.1.2 Transplantation of alginate encapsulated islets

A discussion of the transplantation of encapsulated islets begins with an exploration of the site of implantation of the capsules. The regular sites involved, namely the liver, spleen and kidney capsule have not been considered to be ideal for the transplantation of encapsulated islets due to the large increase in the volume of the graft resulting from the alginate capsule, though one study has reported that the intraportal delivery may be feasible (284). Regardless, larger sites have been favored for implantation of

encapsulated islets, particularly the peritoneal cavity. Unfortunately, the peritoneal cavity has its drawbacks as a site, such as the low oxygen tension (285) and non-physiologic route for insulin delivery (286). Creation of a surgical omental pouch has shown promise as an alternative site to the peritoneum (255), and recently one study involving transplantation of encapsulated islets under the skin and kidney capsule has shown promising results (287), though this has yet to be repeated elsewhere.

As of now, transplantation of encapsulated islets has been extensively studied and has been shown to enhance the survival of both allogeneic and xenogeneic islets in small animal models. Intraperitoneal (i.p.) transplants of encapsulated islet allografts have been performed successfully in mice (288) and rats (289), and intraperitoneal xenografts transplanted into diabetic mice have normalized blood glucose for over 100 days (290;291). Transplantation of encapsulated islet allografts have also shown moderate success in pancreatectomized larger mammals (255). Furthermore, one center has shown sustained insulin independence for more than 9 months after i.p. transplantation of microencapsulated allogeneic islets into spontaneously diabetic dogs (292;293) and in one diabetic human (294), and in another center xenotransplantation of pig islets into cynomolgus monkeys has demonstrated normoglycemia in recipients for up to 803 days in the absence of adjuvant immunosuppressive therapy (295). While insulin independence as this has not been repeated in other labs, recent results demonstrating reduced blood glycemia and glucose requirements in cynomolgus primates transplanted with neonatal porcine islets (296) and two humans transplanted with allogeneic islets (297) demonstrate the clinical relevance of alginate encapsulation of islets and the need for continued study.

#### 1.5.3.1.2.1.3 Obstacles to transplantation of encapsulated islets

Despite successes using encapsulated islets to alleviate hyperglycemia in diabetic animals and humans, this technology is still fraught with areas requiring improvement. Already discussed were concerns with alginate biocompatibility, the emphasis of which cannot be understated, particularly as it pertains to the necessity of complete encapsulation of islets for transplantation. Transplantation of incompletely encapsulated tissue, particularly in humans, would negate the immune barrier properties of this technology. Additionally, capsule size is of concern (298), not only with respect to the volume and site of implantation but also with regards to the diffusion properties of the capsule and the ability of oxygen and other nutrients to reach the islets (299). Furthermore, there are technical difficulties with regards to the relatively slow production rates of capsules (300), which would certainly need to be overcome prior to this therapy becoming widely available to the population of diabetic patients *en mass*.

Technical difficulties associated with islet encapsulation technology are sure to be overcome with time and as methods improve. Of perhaps greater concern are the immunological barriers to islet transplantation that investigators hope to overcome through the use of alginate immunoprotection. Though alginate microencapsulation provides some protection to islets from host-mediated immune destruction, it is by no means a perfect barrier. Observations that transplanted capsules have shown the adherence of cells to the membrane surface indicates that the material forming the capsules was not sufficiently inert to prevent a foreign-body reaction (301;302). As we discussed earlier, alginates of differing compositions exhibit different immunogenicities, a likely reason for difficulties in reproducability of results between labs. In fact, it has

been shown that commercial alginates contain at least 10-20 fractions with different electrophoretic mobility, which induced lymphocyte proliferation *in vitro* (303). Incomplete encapsulation of islets and subsequent capsule fracture and exposure of donor antigens to the host system further complicates the problem, adding another source of immunogenicity. This may be minimized, however, with effective selection of intact islet capsules. Furthermore, it must be remembered that the microcapsule is porous. This means that shed antigens, complement, cytokines, and radicals have the potential of crossing the membrane and eliciting a host immune response or causing destruction of the contained islet. It has been shown that the macrophage is the major cell type interacting with microencapsulated islet grafts (304;305), and that macrophage derived cytokines and other factors are deleterious to β-cell function and integrity (304;306-308). Furthermore, humoral mechanisms may come into play after HAR, and whereas it is highly unlikely that the larger immunoglobulins or immunological cells would come into contact with the encapsulated islets, it has been shown that induction of IgG and IgM antibody production occurs after encapsulated xenotransplantation (309;310;310). Since empty capsules do not initiate a humoral response, it seems that activation of the host's immune system by factors that are released by the encapsulated graft through the semi-permeable membrane may play a crucial role as a trigger of cellular and humoral immunity. Resultant inflammatory infiltrate and microcapsular fibrotic overgrowth clearly impair trans-membrane gas/nutrient fluxes (311;312). Clearly, for all of the emerging successes in encapsulated islet transplantation, researchers must certainly address potential problems prior to clinical implementation of this therapy.

## 1.6 OBJECTIVES & GENERAL THESIS OUTLINE

The primary objective of this work is to take a straight forward, systematic approach to islet encapsulation with respect to using models of increasing immune challenge to explore the viability potential therapy for type-1 diabetes. While much work has been done in the area of islet encapsulation, what is missing from the field is a unified progression of experiments from tissue culture on to animal models of sequentially increasing immunogenicity in order to better understand the shortcomings of this method and thus better know how to overcome deficiencies. Furthermore, while much work has been done in the area of alginate modification and purification, and the subsequent analysis of the products, little has been published on the procedures that have been used, and little has been done to compare how these processes affect graft success when these alginate products are used to encapsulate islets. The hope is that by investigating the alginate, the graft, and the recipient we will have a greater understanding of this technology, overcome the potential barriers and find clinical applicability for islet encapsulation in the foreseeable future.

Chapter 2 begins this thesis by studying the long term tissue culture of islets, comparing islets encapsulated in alginate with non-encapsulated islets free floating in tissue culture. Here we demonstrate that encapsulation improves the survival and function of islets in long term tissue culture. Encapsulated and non-encapsulated islets were subsequently transplanted into mice lacking a functional immune system, in order to demonstrate the ability of these islets to normalize hyperglycemia in these mice. We demonstrated that encapsulation resulted in improved overall survival and function of islets *in vivo*. Furthermore, encapsulated islets showed tighter glucose control when

stimulated with oral glucose than non-encapsulated islets. Finally, in this chapter we demonstrate that encapsulation not only improves the function of islets both *in vitro* and *in vivo*, but that the encapsulation process results in improved survival and tighter glucose control in transplant recipients with far fewer numbers of islets transplanted than recipients of non-encapsulated islets. Our data clearly demonstrates the benefits of islet encapsulation prior to transplantation into animals lacking a functional immune system.

Chapter 3 explores the use of encapsulated islets in a syngeneic immune competent transplant model. The objective was to demonstrate the ability of the capsule itself to avoid immune detection, as tissue from syngeneic recipients is arguably immunologically inert. In order to successfully achieve our objective, it was necessary for us to develop a protocol for the purification of commercially obtained alginate. This chapter involves an in depth description of the methodology involved in this process, and includes an analysis of both the pre- and post-purification products for contamination, molecular weight, and content of the alginate. We relate the improved survival and function of our transplanted islets to our alginate modification analysis to demonstrate the importance of using a highly pure alginate of specific content to achieve graft success when using encapsulated islets to restore normoglycemia to immune-competent diabetic recipients. Recipients of purified alginate encapsulated islets demonstrated better long-term survival, improved graft function and tighter insulin responsiveness to glucose than did recipients transplanted with islets encapsulated with non-modified alginate. Based on these results, modified alginate was used for encapsulation throughout the remainder of our experiments.

In Chapter 4, we explore the use of encapsulated islets transplanted in immune competent xenogeneic recipients. Experiments described here investigate the ability of the capsule to protect the contained xenogeneic islets from host immunity. Results demonstrated that encapsulation of the islets provided only limited protection – such that the graft survived in a number of recipients, though demonstrable *in vivo* function of the graft was not detected. Thus, a second objective of this chapter was to investigate the co-encapsulation of Sertoli cells with islets in order to determine whether the 'nurse cell' properties and immune-modulatory properties of the Sertoli cells could modify this response. It is shown herein that co-encapsulation with Sertoli cells had the ability to improve *in vivo* graft function in many of the recipients over encapsulated islet alone transplant recipients, and that the co-encapsulated Sertoli cell have the ability to abrogate much of the host immune response. Thus, these results demonstrate that while encapsulation is of certain value in islet survival and function, it is also true that additional techniques, such as the addition of Sertoli cells to the capsule, are of importance in staving off the effects of host immunity not easily overcome by that capsule alone.

In summary, this thesis provides a sequential progression of experiments demonstrating the therapeutic potential of encapsulated islet transplantation. While there is more work yet to be done to advance these techniques to the clinical realm, it is promising that this technology, combined with additional immune modulation, may provide a tool for reducing the systemic immunosuppression currently required in clinical islet transplantation. In particular, as support grows for the transplantation of islets from

xenogeneic sources in humans, the encapsulation of these islets in alginate may provide a level or protection and support otherwise not available.

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# CHAPTER 2

# IMPROVED SURVIVAL OF MICROENCAPSULATED ISLETS DURING IN VITRO CULTURE AND ENHANCED METABOLIC FUNCTION POSTTRANSPLANTATION*

## 2.1 INTRODUCTION

Advances in clinical islet transplantation have resulted in increased attention to islet transplantation as a potential therapy in the management of Type 1 diabetes (1). This therapy has not been of use among diabetics at large, however, due to two major obstacles. First, the shortage of cadaveric human pancreases limits the availability of donor tissue available for transplantation. In fact, current protocols require islets from multiple donors to effectively establish normoglycemia in diabetic recipients. Long term culture has been considered as a potential solution for accruing a suitable mass of islets for transplantation from multiple donors (2,3). Unfortunately, long term islet culture often results in a significant loss in islet mass and viability. The use of biocompatible matrices such as sodium alginate have been used as a means of reducing the detrimental effects of long term culture (4-6).

The second barrier to islet transplantation as a widespread therapy is the current need for chronic immunosuppression to prevent graft rejection (7). To overcome this

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hurdle, microencapsulation of allogeneic or xenogeneic islet grafts has been proposed as a means of protecting islets from immune mediated destruction (8-11). An evaluation of the literature reveals, however, that success has so far been very limited and non-reproducible. The inability of encapsulated islet grafts to reproducibly achieve and maintain long-term euglycemia is presumably related not only to insufficient immunoprotection but also to a variety of technical and metabolic issues, including the: encapsulation process, composition/purity of the microcapsule (12), capsule size & surface structure (13,14), poor biocompatibility (15,16), and endotoxin levels of the alginate (17,18). Furthermore, while the addition of poly-L-lysine (PLL) has been used to reduce capsule porosity (19), its addition has resulted in an increased fibrotic response (15). It has been shown also that a simple alginate capsule, devoid of PLL, retains the ability to prolong graft survival and protect the graft (6).

The aim of this study was to determine whether islets, encapsulated in a highly purified calcium-alginate capsule exhibit prolonged survival and function during long term tissue culture and post-transplantation. We used immuno-incompetent mice implanted with canine islets, as well as syngeneic mouse islet grafts where a potential fibrotic response could be examined.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Experimental design

The survival and function of encapsulated canine islets were examined both in vitro and in vivo. Controls included non-encapsulated islets. Islets were cultured for 1,

2, and 3 weeks and were compared with respect to islet recovery, glucose stimulated insulin secretion, and metabolic function following transplantation. Experiments compared the success and longevity of transplants using varying masses of encapsulated or non-encapsulated canine islets in nude mice. Another set of experiments determined the efficacy of encapsulated syngeneic mouse islets transplanted intraperitoneally to assess graft survival in a model able to exhibit an inflammatory response.

### 2.2.2 Animals

Donor pancreases were obtained from halothane anesthetized mongrel dogs (University of Alberta, Edmonton, Alberta, Canada) of either sex, weighing 9-30 kg. Male, inbred, athymic nude BALB/c mice (age 6-8 weeks) (Jackson Laboratories, Bar Harbour, MA, USA) were used as recipients of the canine islets. Male BALB/c mice (University of Alberta) were used as donors and as recipients for syngeneic grafts. Mice were rendered diabetic by intravenous injection of 90 mg/kg body wt alloxan (Sigma Chemical Co., St. Louis, MO) freshly dissolved in 1 mmol/l hydrochloric acid 7-10 days before transplantation. Recipients exhibited blood glucose levels above 20mmol/l. Blood samples were obtained from the tail vein for glucose assay (Medisense glucose meter, Medisense Canada, Mississauga, ON). Animals were maintained under Virus Antibody Free conditions in climatized rooms with free access to sterilized tap water and pelleted food.

### 2.2.3 Preparation and microencapsulation of islets

Canine islets were retrieved through pancreatectomy, islet isolation by collagenase (type V, Sigma) digestion and purification on discontinuous Ficoll (Sigma) density

gradients(20,21). The mean number of islets in each of 6 diameter ranges from 60 to 350μm was determined in order to convert the total yield into 150-μm diameter islet equivalents (IE) (22). Islets were then distributed in groups of 5000 IE per non-treated bacteriologic Petri dish (15 cm diameter; Fisher Scientific, Canada), suspended in 35ml of CMRL 1066 tissue culture medium (Gibco) supplemented with 5.6mmol/l glucose, 2mmol/l L-glutamine, 10% (v/v) fetal calf serum (Gibco), 100 U/ml penicillin and 100μg/ml streptomycin and cultured overnight at 22°C in humidified air (5% $CO_2$, 95% air).

Mouse islets were isolated by collagenase (type V, Sigma) digestion of the pancreas (23), purified using discontinuous Ficoll (Sigma) density gradients, hand-picked and cultured overnight at 22°C in humidified air (5% $CO_2$, 95% air) in supplemented CMRL 1066.

After culture, aliquots of approximately 10,000 canine IE or 3000-5000 mouse islets were washed and re-suspended in 0.44ml with $Ca^{++}$ and $Mg^{++}$ free HBSS (Gibco, Burlington, ON) and mixed with 0.55 ml of 1.5% (w/v) highly purified, high guluronic content alginate (high G alginate, 72% G-content, 28% M-content, 193,800MW, endotoxins 0.25 IU/ml; provided by Metabolex, Inc., Hayward, CA) dissolved in HBSS (no $Ca^{++}$, $Mg^{++}$) (pH 7.4). Alginate microcapsules (250-350μm in diameter) were formed using an electrostatic droplet generator (designed by Metabolex, Inc.) and a 120mM calcium chloride solution for gelation of alginate beads. Capsules were then cultured in supplemented CMRL medium at 22°C for 1, 2, or 3 weeks.

### 2.2.4 Characterization of canine islet preparations after long-term tissue culture

After 1, 2, or 3 weeks culture at 22°C, canine islet preparations were re-counted to determine total IE recovery and assessed for functional viability. These islets were washed in HBSS and transferred to supplemented CMRL 1066 medium for 24h culture at 37°C. The islets' insulin secretory responsiveness to glucose was determined during a 2h static incubation at 37°C (24). Samples of 50 IE were incubated in 24-well non-tissue culture treated plates (Becton Dickson Labware, Franklin Lakes, NJ) with 1.5ml of RPMI medium (Gibco) supplemented with 2mmol/l L-glutamine, 0.5% BSA and either 2.8mmol/l glucose or 20mmol/l glucose. The insulin content of the medium was expressed as a percentage of the total content (i.e., tissue plus medium). Stimulation indices were calculated by dividing the amount of insulin release at 20mmol/l glucose by that released at 2.8mmol/l glucose.

### 2.2.5 Transplantation and metabolic follow-up

In long term culture experiments, non-encapsulated and encapsulated canine islets were transplanted (renal subcapsule and intraperitoneal, respectively) following 1, 2 or 3 weeks culture into Halothane-anesthetized diabetic nude mice. In short term culture experiments, freshly isolated canine islets were cultured for 24 hours, encapsulated, then cultured overnight in supplemented CMRL. Varying masses of islets were transplanted under the renal subcapsule (non-encapsulated) or in the peritoneal cavity (non-encapsulated or encapsulated). To standardize the mass of canine islets transplanted in each experiment, representative aliquots of each preparation were counted prior to implantation to determine total islet equivalents. Grafts consisted of 1000 or 2000 canine

IE for long term culture experiments, while grafts for islet mass studies varied between 250 to 4000 canine IE. For syngeneic mouse grafts, 500 non-encapsulated (renal subcapsular) or encapsulated (intraperitoneally) mouse islets were transplanted into diabetic recipients. All grafts were suspended in supplemented CMRL 1066 medium and then implanted into the peritoneal cavity via a small incision in the linea alba or under the left renal capsule with the aid of a micromanipulator syringe (24).

Mice were monitored for blood glucose levels between 8:00 and 11:00 a.m. When the blood glucose level was ≤8.4mmol/l the graft was deemed a success. At 100-120 days post-transplantation, oral glucose tolerance tests (OGTT) were performed on recipients with normalized basal glycemia and in normal controls. After a 2-h fast, D-glucose (3 mg/g body weight) was administered as a 50% solution by oral gavage into non-anesthetized mice. Blood samples were obtained from the tail vein at 0, 15, 30, 60, and 120min.

At various time points (i.e., >100 days post-transplantation) capsules were recovered by intraperitoneal lavage with phosphate buffered saline containing 12 mmol/l $CaCl_2$. Recovered grafts were subsequently stained with dithizone. To confirm the efficacy of the encapsulated islets at correcting diabetes, the pancreas of each recipient was assayed for insulin content as previously described (24). Animals with renal subcapsular grafts were nephrectomized and subsequently monitored to confirm a return to hyperglycemia.

### 2.2.6 Statistical analysis

Data are expressed as means ± SE of n independent observations. Statistical significance of differences was calculated with a two-tailed unpaired Student's *t*-test or a one-way

analysis of variance in case of multiple comparisons. Significance was attributed when $p<0.05$.

## 2.3 RESULTS

### 2.3.1 Canine islet microencapsulation

Immediately following the encapsulation process, islet recovery was 92±2% (n=10 independent isolations). In eight independent experiments, the volume of representative aliquots of encapsulated canine islets was estimated in 15ml Falcon centrifuge tubes, where as the volume of non-encapsulated preparations was calculated as previously defined (22). The mean volume of 4000 encapsulated and non-encapsulated IE was 160±16µl and 7.2±0.8µl, respectively. When 4000 encapsulated IE were handpicked to eliminate empty capsules the average volume was 150±8µl, demonstrating that approximately 94% of the capsules contained islets.

### 2.3.2 Effect of microencapsulation on canine islet survival and insulin secretory activity in vitro

The number of encapsulated islets recovered throughout the various culture periods was significantly higher when compared to non-encapsulated control islets incubated for the same time (Table 2-1). The recovery of encapsulated islets was 54, 61 and 190% higher than controls following 1, 2, and 3 weeks culture, respectively (Table 2-1). When compared to the start of the experiment, total islet yield of non-encapsulated controls significantly decreased to 63, 55, and 25% after 1, 2, and 3 weeks culture, respectively ($p<0.0001$ at all time points). On the other hand, islet loss was substantially reduced

**Table 2-1**. Effect of microencapsulation on canine islet recovery and insulin secretory activity over a three week period in culture.

| Condition | Islet Recovery (%) | Insulin secretory activity (% content) | | Stimulation indices |
|---|---|---|---|---|
| | | 2.8mmol/L glucose | 20mmol/L glucose | High:low |
| Non-encapsulated | | | | |
| 1 week (n=5) | 63.2±5.3 | 0.55±0.06 | 10.1±0.2 | 18.7±1.8 |
| 2 weeks (n=6) | 55.5±4.2 | 0.97±0.11‡ | 9.4±0.4 | 9.7±0.4‡ |
| 3 weeks (n=4) | 24.5±10.2‡ | 1.07±0.11‡ | 9.2±0.2 | 8.8±0.7‡ |
| Encapsulated | | | | |
| 1 week (n=6) | 97.3±2.6† | 0.43±0.06 | 8.7±0.4 | 20.8±2.0 |
| 2 weeks (n=7) | 89.6±4.2† | 0.54±0.04† | 9.5±0.3 | 17.6±1.4* |
| 3 weeks (n=4) | 71.4±7.6*§ | 0.87±0.04§ | 8.7±0.1 | 10.1±0.4§ |

Values are mean±SEM. Islet recovery was expressed as the percentage of islets remaining after 1, 2 and 3 weeks culture as compared to day 0. Islet insulin release experiments were performed in minimally 4 independent experiments per condition and stimulation indices were calculated dividing amount of insulin release at high glucose (20mmol/L) by that released at low (2.8mmol/L) glucose. *$p<0.05$, †$p<0.001$ vs. non-encapsulated at same time period (unpaired Student's t-test). ‡$p<0.01$ vs. non-encapsulated at 1 week, §$p<0.01$ vs. encapsulated at 1 week (analysis of variance).

when the islets were encapsulated, with 97, 90, and 71% recovery of islet equivalents after 1, 2, and 3 weeks culture, respectively ($p<0.01$).

The insulin secretory activity of control and encapsulated islets was tested by determining the percentages of cellular insulin that was released at low (2.8mmol/l) and high glucose (20mmol/l) during a 2 h static incubation assay. After 1 week culture, no statistically significant differences were observed in the amount of insulin released between the two groups (Table 2-1). Control and encapsulated islets released >18-fold more insulin at high glucose than at low glucose. These values were similar to freshly isolated non-encapsulated canine islets cultured for 24 h at 37°C (0.41±0.02% and 8.9±0.03% at 2.8 and 20 mmol/l glucose, respectively; data not shown). Control islets cultured for 2 or 3 weeks released higher levels ($p<0.001$) of insulin in response to 2.8mmol/l glucose than observed after 1 week culture. Hence, stimulation indices were significantly lower when control islets were cultured for periods longer than 1 week. In contrast, encapsulated islets showed similar insulin secretory responses when cultured for 1 or 2 weeks (Table 2-1), while increased insulin release at low glucose was observed only at 3 weeks culture. Thus, for our encapsulated islets, the calculated stimulation index dropped significantly at 3 weeks culture.

### 2.3.3 Transplantation of long-term cultured canine islets

Control or encapsulated canine islets preserved for 1, 2, and 3 weeks at 22°C were transplanted either under the renal capsule or intraperitoneally into diabetic nude mice, respectively. Transplantation under the renal capsule of 2000 non-encapsulated control IE cultured for 1 week corrected diabetes in two of three recipients for >100 days, whereas one of three recipients maintained normoglycemia for >100 days when culture

**Table 2-2.** Effect of long-term culture on the survival of canine islet grafts in diabetic nude mice.

| Graft | Culture period (weeks) | Days of euglycemia (MST±SE) | % Survival (>100 days) |
|---|---|---|---|
| Non-encapsulated* | | | |
| 2000I.E.'s | 1 | 0, 134x2‡ (89±45) | 66 |
| | 2 | 0, 80, 201‡ (94±58) | 33 |
| | 3 | 0, 23, 312‡ (112±100) | 33 |
| Encapsulated† | | | |
| 1000 I.E.'s | 1 | 134‡, 169§, 170§, 223§ (174±18) | 100 |
| | 2 | 24, 107, 198x2‡ (132±42) | 75 |
| | 3 | 147, 171§, 190‡ (169±12) | 100 |
| 2000 I.E.'s | 1 | 109§, 134‡, 170‡, 198x2‡, 255‡ (177±21) | 100 |
| | 2 | 180x2§, 185§ (181±2) | 100 |
| | 3 | 162‡, 190‡, 248‡ (200±25) | 100 |

Euglycemia defined as blood glucose values ≤8.4 mmol/L. I.E., islet equivalents; MST, mean standard time. *Renal subcapsular transplants. †Intraperitoneal transplants. ‡Grafts recovered from normoglycemic recipients for morphological assessment. §Animal died when normoglycemic.

was prolonged to 2 or 3 weeks (Table 2-2). In contrast, all animals implanted intraperitoneally with 2000 encapsulated islets cultured for 1 to 3 weeks exhibited blood glucose values ≤8.4mmol/l for >100 days (Table 2-2). When 1000 encapsulated islets cultured for 1, 2, and 3 weeks were transplanted, 100% (4/4), 75% (3/4), and 100% (3/3) of the recipients achieved euglycemia for >100 days. At 100 days post-transplantation, OGTTs were performed in aged-matched normal controls and intraperitoneal recipients of 1000 encapsulated islets. OGTTs were not performed in animals implanted with non-encapsulated grafts due to the poor survival past 100 days. Compared to controls, animals receiving 1000 encapsulated islets cultured for 1, 2, and 3 weeks exhibited similar glycemic values at all time points (Figure 2-1). Mice transplanted with canine islets cultured for 3 weeks exhibited mean blood glucose values at times 15 min and 30 min that were significantly higher than those in animals implanted with grafts cultured for 1 week. In all groups, blood glycemia values at min 120 were not significantly different from the values at min 0. The pancreatic insulin content of all recipients of intraperitoneal implants was <2% (0.49±.03μg) that of normal control animals (38.9±1.8μg), indicating that the grafts were responsible for the euglycemia. Similarly, removal of the graft-bearing kidneys from normoglycemic animals was followed by a rapid return to the diabetic state.

### 2.3.4 Effect of canine islet mass and transplant site

In another series of experiments the minimal mass of 48 h cultured non-encapsulated and encapsulated islets required to reverse diabetes in nude mice was assessed following transplantation under the kidney capsule or intraperitoneally. Implantation of 1000 and 2000 non-encapsulated islets under the renal subcapsular space corrected basal glucose

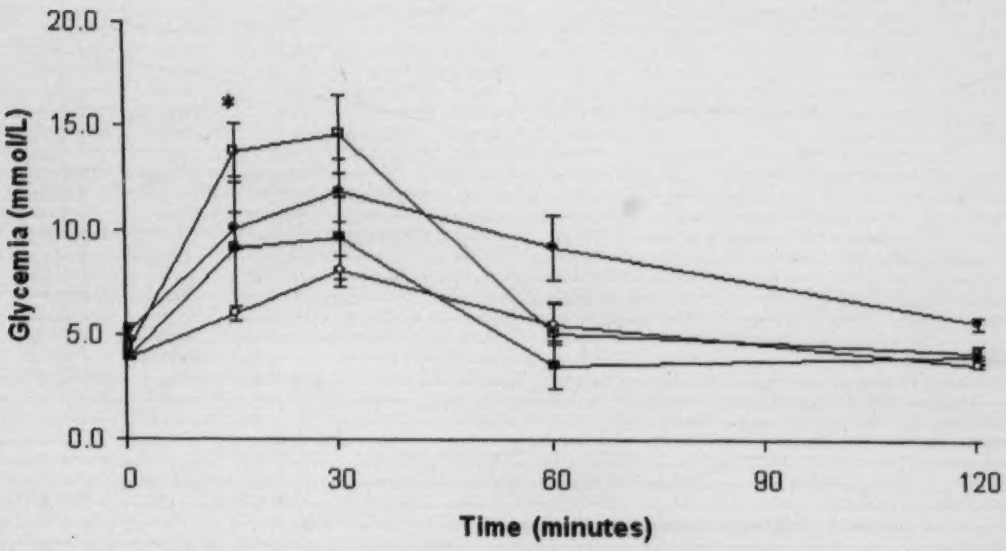


**Figure 2-1**. Blood glucose values during an OGTT in nude mice transplanted with 1000 encapsulated canine islets cultured for 1 (○ ; n=4), 2 (■ ; n=3), and 3 (□ ; n=3) weeks. Values are compared to aged matched normal controls (● ; n=9) and expressed as means ± SEM. Statistical significance of differences was calculated by one-way analysis of variance. *$p<0.05$ vs. 1 week culture.

levels in 50% (4/8) and 83% (15/18) of the animals, respectively (Table 2-3). However, at >100 days post-transplantation, euglycemia remained in only 2/8 (25%) and 12/18 (67%) of the 1000- and 2000-islet group, respectively. Intraperitoneal transplantation of either 2000 or 4000 non-encapsulated islets had little effect at correcting diabetes, as <15% of the animals exhibited normoglycemia lasting only a short time (Table 2-3). In contrast, normalization of blood glucose occurred in 100% of recipients receiving intraperitoneal grafts of 1000, 2000, and 4000 encapsulated islets (Table 2-3). In these

**Table 2-3.** Metabolic follow-up of non-encapsulated and encapsulated canine islet grafts in diabetic nude mice.

| Group | Islet mass (I.E.) | n | Days of euglycemia (MST±SE) | % Survival (>100 days) |
|---|---|---|---|---|
| Non-encapsulated | | | | |
| kidney | 1000 | 8 | 0x4, 1, 10†, 120*, 163 (>37±23) | 25 |
| | 2000 | 18 | 0x3, 46, 66, 85, 114, 116x3*, 120x2*, 133x2*, 156x2*, 184x2* (>103±14) | 67 |
| i.p. | 2000 | 9 | 0x8, 6x1 (1.0±1.0¶) | 0 |
| | 4000 | 8 | 0x7, 37 (5±5¶) | 0 |
| Encapsulated | | | | |
| i.p. | 250 | 8 | 0, 11, 12, 38x2, 87, 122x2* (>54±18) | 25 |
| | 500 | 18 | 0x3, 23, 50, 135, 137x4*, 170x3*, 184*, 217*, 262†, 280*, 328* (>141±23‡¶) | 72 |
| | 1000 | 19 | 79, 109, 132, 135, 136x3†, 137x5*, 150*, 177*, 184x4*, 223† (>149±8‡¶) | 95 |
| | 2000 | 28 | 28, 48, 108, 116x5*, 120x6*, 137x4*, 148, 184x4*, 257*, 262x2*, 267x2 (>146±12‡¶) | 93 |
| | 4000 | 11 | 104, 116x4*, 102x2*, 133x2*, 149x2* (>124±4‡§) | 100 |

Euglycemia defined as blood glucose values ≤8.4 mmol/L. I.E., islet equivalents; MST, mean standard time; i.p. intraperitoneal. *Grafts recovered from normoglycemic recipients for morphological assessment. †Animal died while normoglycemic. Statistical significance of differences between groups were calculated by factorial two-way analysis of variance. ‡$p<0.01$ vs. non-encapsulated kidney (1000 I.E.). §$p<0.05$. ¶$p<0.01$ vs. non-encapsulated kidney (2000 I.E.).

three groups, basal normoglycemia was maintained in 95%, 93%, and 100% of the animals for >100 days post-transplant. When the mass of the intraperitoneal grafts was decreased to 500 and 250 encapsulated islets, 72% (13/18) and 25% (2/8) of the recipients maintained euglycemia for periods greater than 100 days, respectively.

Glucose tolerance tests were performed in normoglycemic recipients and non-diabetic control mice at 100 days post-transplant (Figure 2-2). Compared to age-matched

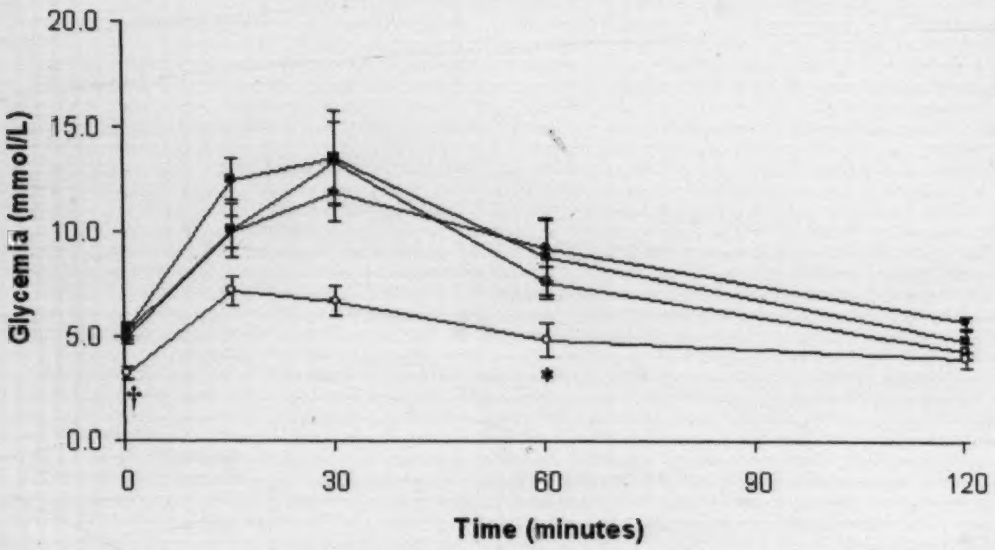


**Figure 2-2**. Blood glucose values during an OGTT in nude mice transplanted with 2000 non-encapsulated canine islets under the kidney capsule (○ ; n=5) or 500 (■ ; n=7) and 1000 (□ ; n=10) encapsulated canine islets in the intraperitoneal cavity. Values are compared to aged matched normal controls (● ; n=9) and expressed as means ± SEM. Statistical significance of differences was calculated by one-way analysis of variance. *$p<0.05$, †$p<0.01$ vs. control.

normal controls, animals implanted with 2000 non-encapsulated islets under the kidney capsule showed significantly lower ($p<0.01$) glucose values at 0, 30, and 60 min during the OGTT. When recipients of 500, 1000, 2000, and 4000 encapsulated islets placed intraperitoneally were compared, their blood glucose levels exhibited similar values to each other and to age matched normal controls, and were not statistically different at all time points during the OGTT (Figure 2-2; data represents recipients of only 500 and 1000 encapsulated islets). When the 2000 non-encapsulated (kidney capsule) group was compared to recipients of encapsulated islets, their glucoses were significantly lower at 0 min ($p<0.05$ vs. 500, 1000 and 2000 islets), 15 min ($p<0.01$ vs. 500 islets), and at 30 min ($p<0.05$ vs. 500, 1000, and 2000 islets). In all groups, the glycemia at min 120 was not significantly different from values at min 0.

Examination of recovered encapsulated canine islets at ≥100 days post-transplantation revealed structurally intact capsules and islets with no signs of capsule breakage or fibrotic reaction on the capsules' surface (Figure 2-3). In all the recipients of non-encapsulated islets, removal of the graft bearing kidney was followed by a rapid return to the diabetic state. The pancreatic insulin content of recipients of encapsulated islets was <2% (<0.6 μg) that of normal nude mice (38.9±1.8 μg). These results demonstrate that the normoglycemia observed in these recipients was attributable to insulin production from the encapsulated islet grafts and not from residual pancreatic beta cells.

### 2.3.5 Syngeneic transplantation of mouse islets

Syngeneic BALB/c mouse islet grafts were transplanted to test the ability of encapsulated islets to normalize the diabetic state in immunocompetent animals in order to observe the

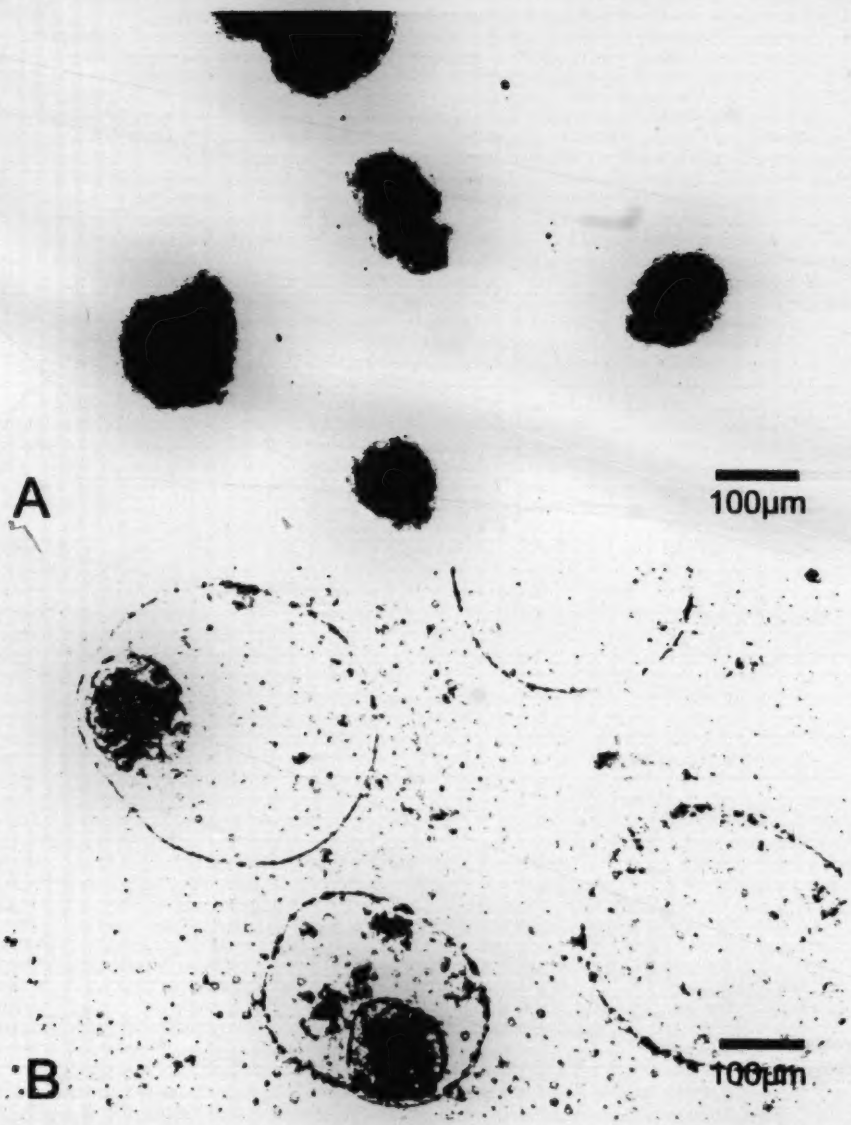


**Figure 2-3.** Light micrographs of dithizone stained encapsulated canine islets before (A) and after (B) implantation into the peritoneum of alloxan-induced diabetic nude mice.

biocompatibility of our alginate. All animals implanted with either 500 non-encapsulated (kidney capsule) or encapsulated (intraperitoneally) islets exhibited normalization of blood glucose levels (<8.4mmol/l) within 48 h post-transplant (Figure 2-4A). This metabolic state was maintained over the 150 day follow-up period and there were no significant differences between the two groups (Figure 2-4A). An OGTT was performed on normoglycemic recipients and age matched normal controls approximately 150 days post-transplant (Figure 2-4B). Compared to normal controls, animals implanted with either non-encapsulated (kidney capsule) or encapsulated (intraperitoneally) islets showed comparable glucose values at all time points throughout the OGTT. Furthermore, at the end of the study removal of the graft bearing kidney resulted in a rapid return to hyperglycemia, whereas all pancreases from encapsulated islet recipients contained negligible insulin (<0.5 μg).

## 2.4 DISCUSSION

This study examined whether microencapsulation with a highly-purified alginate preserves islet survival and function during long term tissue culture, as well as following transplantation into both immune competent and immune deficient animals. Initial experiments assessed canine islet survival and insulin secretory responsiveness after long-term tissue culture. Once these experiments demonstrated excellent function and survival in vitro, encapsulated canine islets were then transplanted in diabetic nude mice to determine metabolic function and the minimal mass required to achieve euglycemia. An immuno-incompetent nude mouse model was chosen in order to exclude the effects of rejection, thereby accurately examining the metabolic function of the islet grafts. Syngeneic mouse islet transplants were also conducted to test the biocompatibility of the grafts and the ability to achieve euglycemia and glucose tolerance. Using a syngeneic

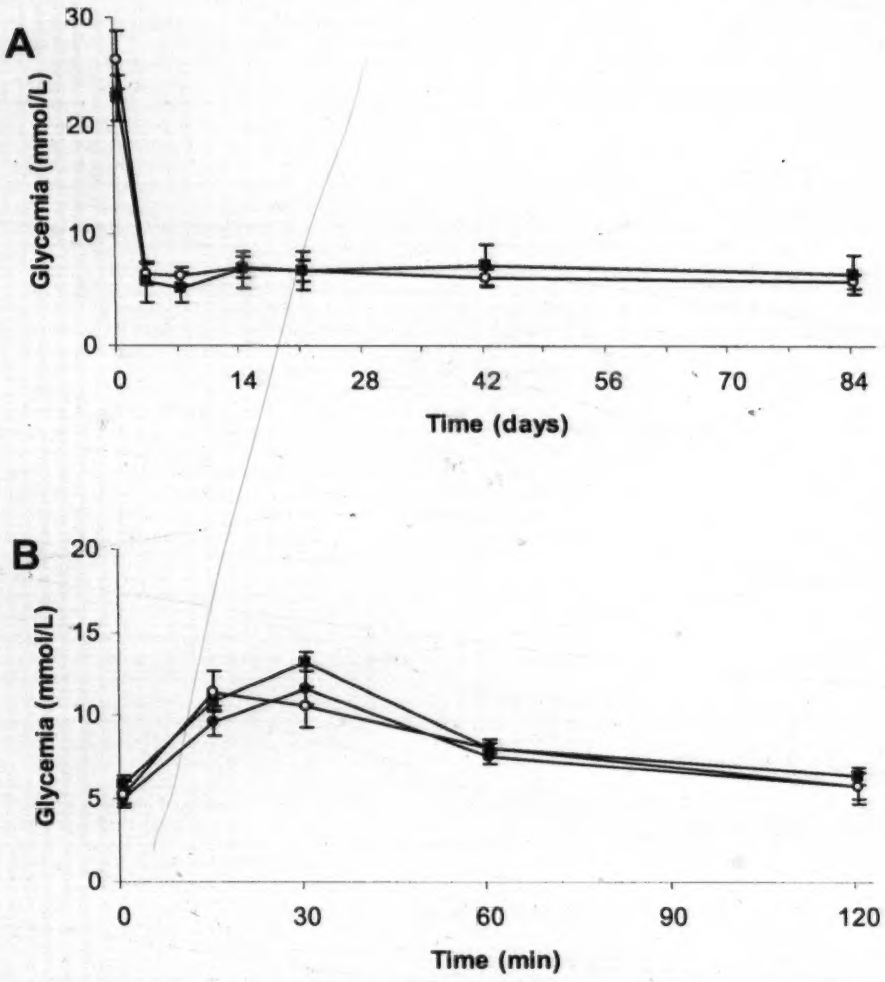


**Figure 2-4**. Function of syngeneic pancreatic islet grafts in alloxan-induced diabetic BALB/c mice. Grafts consisted of 500 non-encapsulated (○ ; n=6; kidney capsule) or microencapsulated (■ ; n=11; intraperitoneal) syngeneic islets. (A) Non-fasting blood glucose concentrations are shown from the time of transplantation (day 0) to the conclusion of the follow-up period. (B) Blood glucose values during an OGTT in normal control BALB/c mice (● ; n=5) and those transplanted with 500 non-encapsulated (○ ; n=6; kidney) or encapsulated (■ ; n=7; intraperitoneal) syngeneic islets. Values are expressed as means ± SEM.

model allowed us to observe any potential effects the alginate might have on the graft given that syngeneic tissue would not specifically elicit an immune response.

When non-encapsulated canine islets were cultured at 22°C, islet recovery decreased significantly and when the culture period was extended to 2 and 3 weeks, recovery of non-encapsulated canine islets was further reduced. However, when canine islets were encapsulated islet loss during culture was much lower with recoveries of 70% after 3 weeks. After these culture periods, glucose stimulated insulin secretion was assessed. During the static incubation at 2.8mmol/L and 20mmol/L glucose, similar amounts of insulin were secreted from both non-encapsulated and encapsulated islets cultured for 1 week. Moreover, both non-encapsulated and encapsulated canine islets maintained their glucose responsiveness after 1 week at 22°C since their insulin secretory activity remained comparable to freshly isolated islets (data not shown). After 2 and 3 weeks culture, the insulin secretion at high glucose was comparable; however the calculated stimulation indices for non-encapsulated islets were significantly decreased. This lower response was a direct result of higher basal release of insulin at low glucose (2.8mmol/L) which would suggest that extended culture induces beta cell injury causing passive discharge of insulin and ultimately an abnormal secretory capacity. This reduction of islet function was also observed by the poor ability of non-encapsulated islets cultured for 2 or 3 weeks to correct diabetes in nude mice, whereas encapsulated islets cultured for up to 3 weeks achieved euglycemia and normal glucose tolerance in 75% to 100% of the animals for ≥100 days.

This comparative study on recovery and function of long-term cultured canine islets demonstrates that microencapsulation with highly purified alginate can be

successfully used for storage of islet grafts for prolonged periods without a significant loss of islet cell mass or function. This technique maintains higher islet yields and functional viability both in vitro and post-transplantation than conventional suspension tissue culture. Microencapsulation may achieve this protective effect by providing an environment mimicking the anatomical three-dimensional pancreatic matrix structure, thus preventing islet deterioration and preserving survival and functional viability. Other studies have similarly shown that extracellular matrices play a significant role in inducing islet growth and differentiation (25-27). Preservation of individual islets within a microcapsule also offers the advantage over conventional culture by preventing islet aggregation which often causes central necrosis of these large cellular aggregates. Furthermore, the ability to maintain islets in long-term culture is useful for clinical islet transplantation since it permits the combination of isolates from multiple donors in order to achieve a critical beta cell mass.

We also compared the success and longevity of varying masses of encapsulated or non-encapsulated canine islets. Transplantation of 1000 non-encapsulated canine islets renal subcapsularly failed to correct hyperglycemia in the majority of recipients; whereas, 83% of the animals achieved normoglycemia when the mass was increased to 2000 islets. In contrast, when 2000 or 4000 non-encapsulated islets were implanted intraperitoneally, only one of seventeen recipients exhibited normoglycemia for longer than a day. This latter observation was expected because it has been previously reported that the intra-peritoneum is not a very effective site for non-encapsulated islet grafts (28,29). In fact, intraperitoneal transplantation with even encapsulated islets has in the past shown problems with cellular overgrowth of the capsules (30), immune system sensitization

(31), and an inability of encapsulated islets to sense glucose concentration changes (32). In the present study, long-term normoglycemia was however, obtained when ≥500 encapsulated islets were transplanted into the peritoneum. The majority of these recipients remained euglycemic for ≥100 days and exhibited comparable glucose tolerance curves to normal controls. To our knowledge, this is the first report demonstrating that encapsulated islets transplanted intraperitoneally can not only reproducibly achieve euglycemia but also normal glucose tolerance.

Finally, grafts consisting of 500 encapsulated BALB/c mouse islets also reproducibly achieved euglycemia for 150 days post-transplantation. Blood glucose values for these animals were not significantly different than those obtained from animals transplanted with non-encapsulated syngeneic islets. Furthermore, both of these groups of animals showed normal glucose tolerance during an OGTT at 150 days when compared with our age matched controls. Since syngeneic islets will elicit no host response, and given that there were no appreciable differences between our groups, we concluded that the alginate used in these experiments is biocompatible since no detrimental effect was observed.

In all of the above experiments, the encapsulated grafts were shown to exhibit no fibrotic overgrowth after ≥100 days post-transplant. Much of the overgrowth of fibroblasts on capsules in other studies can be attributed to the effects of poly-L-lysine (15), however the complete absence of fibroblasts on the alginate capsules in our experiments may be a result of the high purity of the alginate, thereby contributing to its improved biocompatibility. This may also explain why a lower mass of encapsulated canine islets placed intraperitoneally (i.e., 1000 islets) was more effective at achieving

normoglycemia as compared to non-encapsulated control islets transplanted under the kidney capsule (i.e. 2000 islets). Since islets placed under the kidney capsule tend to fuse together forming large clumps, these grafts maybe exposed to more hypoxic conditions resulting in necrosis and/or apoptosis (33). Furthermore, encapsulation of islets, while not providing a perfect immune barrier, certainly may prolong graft survival (6,34). While the exclusion of poly-L-lysine from our alginate capsule increases the porosity of the capsule (35,36), the capsule retains the ability to prevent contact of host cells such as macrophages with the islets, ultimately extending graft survival (37). Nonetheless, an open pore alginate capsule is not an immune barrier so much as it is a support matrix to enhance islet viability, which is a critical step needed before a perm-selective membrane is placed on the islet. The ability of these capsules to so successfully restore glucose responsiveness may in large measure be a result of the high purity and low endotoxin content of the alginate (6,34). Therefore, microencapsulating islets with an alginate of excellent biocompatibility can provide an environment that supports long-term islet graft survival and function, ultimately reducing beta cell loss and dysfunction in the immediate post-transplant period.

This study demonstrates that a purified alginate can be successfully used for long-term storage of islet grafts. This approach succeeds in maintaining higher islet yields and functional viability than conventional suspension tissue culture. It is also encouraging that transplantation of islet grafts microencapsulated with purified alginate into a non-vascularized site such as the peritoneal cavity permits the grafts not only correct basal glycemia but also achieve normal glucose tolerance. Furthermore, it is encouraging that

these grafts have the ability to provide long term reversal of hyperglycemia without any observable host reaction to the alginate.

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# CHAPTER 3

# ALGINATE MODIFICATION IMPROVES LONG TERM SURVIVAL AND FUNCTION OF TRANSPLANTED ENCAPSULATED ISLETS

## 3.1 INTRODUCTION

Clinical pancreatic islet transplantation has been reported to normalize blood glucose and lead to insulin independence in patients receiving this therapy (1;2). Despite its successes, however, it has been recently reported that only about 10% of these patients remain insulin independent for five years, and that much still needs to be done to maintain islet function and reduce the toxic immunosuppression currently used to prevent graft rejection (3). In an attempt to alleviate the need for systemic immunosuppression, microencapsulation has been examined for its potential to isolate and protect islets from host immunity, and to provide a microenvironment mimicking that of the native pancreas (4-6). This technology has been fraught with varying and inconsistent results over the years, largely due to the great variability in the alginate used by the different groups (7;8). Alginate microcapsules may vary in size (9), composition (proportion of mannuronate (M) and guluronate (G) blocks) (10;11), or purity (presence of endotoxins, proteins, polyphenols) (7;12-14). Furthermore, there are differences in the choice of cross-linking ions (usually calcium or barium), and of the presence or absence of additional coating layers such as poly-L-lysine (PLL) (15;16). These factors and others

lead to varying degrees of success or failure of the encapsulated islets. For example, the capsular material may in some way be toxic to the contained islets. Some have observed overgrowth of the capsules, primarily by macrophages (17), which may again be the result of the alginate or of antigens shed by the islets within. Whatever the case may be, it is critical that these factors be identified to develop a better alginate and a better capsule for use in the research arena and in the clinic.

The goal of this study was to develop a method to purify commercially available alginate for use in islet transplantation. Furthermore, we wanted to observe and compare islets encapsulated in our pre- and post-purification alginates on their ability to normalize blood glucose and protect these grafts from rejection. Finally, we hoped that observation and analysis of grafts recovered from our recipients at various time points might give us a better understanding of the mechanisms involved in graft failure and give us some idea of how purification might provide us with alginate more suitable for use in a clinical setting.

## 3.2 RESEARCH DESIGN AND METHODS

### 3.2.1 Animals

Male BALB/c mice aged 6-10 weeks (HSLAS, Edmonton, AB) were used as both donors and recipients for transplantation. Diabetes was induced by intraperitoneal (i.p.) injection of streptozotocin (STZ; 275mg/kg body weight, freshly dissolved in acetate buffer, pH4.5; Sigma Chemical Co., St. Louis, MO) two days prior to transplantation. Diabetes was confirmed by the presence of hyperglycemia (18.0mmol/L). Blood was obtained from a snipped tail and blood glucose levels were monitored with a portable glucose

meter (One Touch Ultra, Johnson & Johnson, Milpitas, CA). The animals were kept under conventional conditions in climatized rooms with free access to tap water and standard pelleted food in accordance with the recommendations of the Canadian Council on Animal Care.

### 3.2.2 Islet isolation

Islets were isolated from male BALB/c mice aged 6-10 weeks (18). Mice were put down with halothane then anaesthetized with 0.4mL of l 2,2,2-tribromoethanol (10mg/ml; Sigma Chemical Co., St. Louis, MO). Each mouse was laid in its back, had its belly soaked with 70% ethanol, and had a full length midline incision cut from the groin to past the breastbone with a small scissors and toothed forceps. The skin was cut first, following which the abdomen was opened by cutting through the muscle layer. The mouse was immobilized on a mouse board with skin anchors. Using a 3mL syringe, 2.5mL collagenase solution (1mg/mL; Sigma) was drawn up. A 27G needle was attached to the syringe, following which the needle was bent to a 45° angle, bevel inside. A hemostat was attached to the common bile duct at its point of attachment to the small intestine. The lobes of the liver were swept back to expose the bifurcation in the common bile duct, at which point we cannulated the bile duct with the syringe and needle to slowly inject all of the collagenase solution. After removing the syringe and needle, the heart was clipped, the pancreas was carefully excised away from all of the connective tissue and the perfused pancreas was dropped into a tube of 30mL Hank's Balanced Salt Solution (HBSS) on ice. This was repeated for each of the animals, with all of the pancreases collected into one of two tubes of 30mL HBSS on ice (in staggered order into each tube).

The harvested pancreases were then removed from the HBSS in exchange for tubes of collagenase solution (1mg/mL). These tubes were then capped and placed in a shaking water bath for approximately 13 minutes (digestion times may vary, however, between 9 to 14 minutes). Next, the tubes were removed from the bath, given a brief hand-shake and topped up with cold HBSS to stop the digestion. The tubes were centrifuged at 1500rpm for 30 seconds. The supernatant was aspirated, and the pellet resuspended in 15mL HBSS and vortexed. The resuspended tissue was poured through a filter funnel (nylon monofilament screening fabric, 500 micron mesh opening, Sefar Canada Inc., Scarborough, ON) into a 50mL tube. The digestion tubes were rinsed with HBSS and the rinse also was poured through the filter funnel into the collection tube. The filter was rinsed finally using 10mL of HBSS in a 10mL syringe with a 23G needle to wash any other islets into the collection tube. The collected islets were separated into tubes with approximately 2.5 pancreases per tube. The tubes were then topped up with cold HBSS, capped and centrifuged at 1500rpm for 30 seconds. The tubes were again aspirated down to the pellet, followed by the addition of 10mL of 25% Ficoll (Sigma Chemical Co., St. Louis, MO) to each of the tubes. The tubes were capped and vortexed to suspend all of the tissue. Then, using a 10mL pipette, 6mL of each of 23.5%, 21.5% and 11.5% Ficoll were layered on top one another to form a discontinuous gradient. The tubes were centrifuged at 2000rpm for 10 minutes (brake off) and the islets picked off of the first gradient interface using the same 14G stainless steel cannula for each tube. Islets were collected into clean tubes containing 20mL HBSS (half as many tubes as used to do Ficoll gradients) and centrifuged at 2000rpm for 1 minute. The tubes were aspirated and washed a second time in HBSS, following which a second gradient was done, this time

centrifuged for 2000 rmp for 2 minutes (brake off). Using the same cannula as before, the islets were again picked off of the first interface and placed in the last wash tubes for two washes as before. Following the final wash, the supernatant was aspirated and the pellet resuspended in 10mL M199 (Gibco BRL, Life Technologies, Burlington, ON) and poured into a Petri dish. The tube was rinsed with M199 and added to the Petri dish. Using a Pasteur pipette connected to the hand pipettor, all of the islets were picked out and transferred to another dish and cultured in Ham's F-10 culture media (Sigma Chemical Co., St. Louis, MO).

### 3.2.3 Alginate Purification

The purification process began with bleaching 4% w/v activated charcoal (JT Baker, Phillipsburg, NJ) in 0.1M sodium perchlorate (Fisher Scientific, Fair Lawn, NJ) for 30 minutes, followed with 2 washes in distilled water and 2 washes in 95% ethanol. The charcoal was allowed to dry overnight. Alginate powder (Pronova UP-MVG, Pronova Biomedical, Oslo, Norway; batch 1 (Pronova 701-256-08) and batch 2 (Pronova FP-301-02) was purified by first dissolving in distilled deionized water (10g/L). Bleached activated charcoal (20g/L) was added and the mixture stirred for 3 hours. Removal of the charcoal through a succession of filters of decreasing porosity from 1.2 microns, to 0.45 microns, to 0.22 microns and down to 0.10 microns. The solution was brought to neutral pH, following which we added magnesium chloride (20g/L) and stirred for 30 minutes. Next a 1.7% calcium chloride solution (26mL/L) was added, stirring for another 30 minutes. The precipitated alginate was recovered then re-dissolved in 0.1M EDTA + 10mM Hepes buffer (pH 7) at a concentration 75mL per gram of original alginate dissolved. Once dissolved, 0.125g NaCl per gram of alginate and stirred for 30 minutes.

This solution was then run at 3,000 x g using 8,000 NMWL Centricon Plus-80 ultra-filtration tubes (Millipore Corporation, Bedford MA), discarding the distillate. Then, 60% ethanol was added to this final solution, allowed it to settle refrigerated at 4°C for 3 hours. The mixture was centrifuged at 2000rpm at 4°C for 10 minutes to pellet the alginate. The supernatant was removed and the process was repeated 5 times more with increasing concentrations of ethanol (60%, 80% x 2, and 100% x 3), each time at 4°C. To dry the final precipitate, alginate was spread in a Buchner funnel set-up in a hood heated to 35°C, allowing the alginate to dry overnight. Alginate was then prepared as a 1.5%w/w solution (pH 7) in HBSS without magnesium or calcium (Gibco) filtered through a Gelman "Serum Acrodisc" (Pall Life Sciences, Ann Arbor, MI) 37mm syringe filter (0.2 micron) and stored at 4°C for later use. Two batches of alginate were purified from batches of Pronova UP-MVG to ensure reproducibility of the modification process, though only alginate from the first batch has been used for the islet encapsulation and transplantation studies in this experiment.

### 3.2.4 Analysis and composition of alginates

Purified and non-purified batches of alginate were sent to Dr. Berit Strand (Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway) for analysis. The composition of original and purified alginates was determined by measuring the presence of mannuronic (M) and guluronic (G) acid as analyzed by $^{1}$H-NMR (nuclear magnetic resonance) spectroscopy (300 MHz, Bruker DPX), with fractions of dimers and trimers calculated(19). Average molecular weights of the alginates were measured by size exclusion chromatography–multi-angle laser light

scattering (SEC-MALLS). Endotoxin levels in the samples were measured using a *Limulus amebocyte* lysate assay (Chromogenix AB, Mölndal, Sweden).

### 3.2.5 Islet encapsulation

With hood temperature set to 30°C and solutions at room temperature, we begun the encapsulation process by washing islets (~10,000 IE per encapsulation) several times with calcium and magnesium free HBSS, centrifuging at 1500rpm for 1 minute between washings. Following the final wash, the tissue was centrifuge and the supernatant aspirated. The tissue was then resuspended into 0.45mL of HBSS (no $Ca^{++}$, $Mg^{++}$), to which we added 0.45mL of the 1.5% alginate solution (either Pronova UP-MVG or our purified alginate, both batch 1) and lightly mix until homogeneous. The alginate cell suspension mixture was then run through an electrostatic pump generator (designed by Metabolex, Hayward CA) across a voltage potential into a 120mM calcium chloride solution causing gelling of the formed alginate beads 400-500μm in diameter. These beads were washed several times with HBSS (no $Ca^{++}$, $Mg^{++}$), then twice more with Hams F-10 culture media before plating in Hams F-10 culture media and incubating at 37°C.

### 3.2.6 Transplantation of islets

Following 24 hours culture at 37°C in Hams F-10 culture media, encapsulated islets were washed twice in HBSS, then suspended in Hams F-10. Prior to implantation, in order to standardize the mass of mouse islets transplanted in each recipient, representative aliquots of each preparation were counted and sized to determine total islet equivalents (IE). Graft mass varied between 900 to 1200 mouse islet equivalents. Encapsulated

islets were then implanted into the peritoneal cavity with an 18 gauge cannula via a small incision in the *linea alba*. The incision was closed using MikRon Autoclip stainless steel wound clips (9mm; Becton Dickson, Sparks, MD).

### 3.2.7 Follow-up and oral glucose tolerance test

Mice were monitored for blood glucose levels between 8:00 and 11:00 a.m. When the blood glucose level was ≤8.7 mmol/l the graft was deemed a success. At 105 days posttransplantation, an oral glucose tolerance test (OGTT) was performed on recipients with normalized basal glycemia and in normal controls. After a 2-h fast, D-glucose (3 mg/g body weight) was administered as a 50% solution by oral gavage into non-anesthetized mice. Blood samples were obtained from the tail vein at 0, 15, 30, 60, and 120 min. Total area under the curve (AUC) for glucose clearance was calculated with the trapezoidal rule method for the purposes of comparison of OGTT curves.

### 3.2.8 Graft removal and histology (Microcapsule explantation)

At various time points (i.e., >100 days posttransplantation) capsules were recovered by an intraperitoneal lavage with phosphate buffered saline containing 120 mmol/L $CaCl_2$. From the recovered grafts, a small sample was taken and stained with dithizone (Sigma) for observation. Islets were observed for the presence or absence of necrosis and for general observation of the tissue. Capsules were observed for shape, size and for the presence or absence of breakage and/or fibrotic overgrowth. To confirm the efficacy of the encapsulated islets at correcting diabetes, the pancreas of each recipient was assayed for insulin content as previously described.

### 3.2.9 Assessment of insulin secretion by static incubation

Of the remaining recovered capsules, we assessed the insulin secretory responsiveness of the encapsulated islets during a 2 h static incubation at 37°C. The recovered encapsulated islets were washed twice in HBSS, then once in Hams F-10 culture media. The tissue was separated into two samples, each sample incubated in 1.5mL Hams F-10 supplemented with 2 mmol/L L-glutamine, 0.5% BSA and either 2.8 mmol/L glucose or 20 mmol/L glucose. Following the 2 hour incubation at 37°C the supernatant was collected and evaluated for insulin content by radioimmunoassay (RIA) by the special investigations laboratory at the University of Alberta Hospital. The tissue was collected, washed twice for 10 minutes at 37°C with dissociation media to dissolve the capsules, following which the tissue was also assayed for total insulin content. The insulin content of the medium was expressed as a percentage of the total content (i.e., tissue plus medium). Stimulation indices were calculated by dividing the amount of insulin release at 20 mmol/l glucose by that released at 2.8 mmol/l glucose.

### 3.2.10 Statistical analysis

Data are expressed as means ± SEM of *n* independent observances. Statistical significance of differences was calculated with a two-tailed unpaired Student's *t* test or one-way analysis of variance (ANOVA) in the event of multiple comparisons. Findings were deemed significant at a *p* value of less than 0.05.

## 3.3 RESULTS

### 3.3.1 Alginate purification

Analysis of the two alginate samples by SEC-MALLS demonstrated a reduction in the average molecular weight of the first batch of alginate from 241kDa to 218kDa and the second batch from 242kDa to 220kDa with purification (Table 3-1). Endotoxin levels, as measured by an LAL test, were basically unchanged (Table 3-1). The purification process led to an apparent shift in the composition of the alginate, with the proportion of guluronic acid ($F_G$) in the alginate as measured by $^1$H-NMR spectroscopy increasing with both batches compared with non-purified products (Table 3-1). Similarly, the fraction of guluronate dimers ($F_{GG}$) and trimers ($F_{GGG}$) increased with in both instances. Overall, the process led to an increase in the average length of guluronic acid blocks from 12 to 15 with the first batch and 14 to 21 with the second batch. The fraction of mannuronic acid

**Table 3-1.** Chemical composition and sequence of alginates

| Batch | Sample | $F_G$ | $F_M$ | $F_{GG}$ | $F_{MG}/F_{GM}$ | $F_{MM}$ | $F_{MGG}/F_{GGM}$ | $F_{MGM}$ | $F_{GGG}$ | $N_{G>1}$ | MW | Endotoxin (IU/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Non-purified | 0.68 | 0.32 | 0.57 | 0.11 | 0.21 | 0.05 | 0.077 | 0.52 | 12 | 240900 | 0.24 |
| | Purified | 0.75 | 0.25 | 0.65 | 0.094 | 0.16 | 0.045 | 0.065 | 0.61 | 15 | 217700 | 0.25 |
| 2 | Non-purified | 0.68 | 0.32 | 0.57 | 0.11 | 0.20 | 0.043 | 0.081 | 0.53 | 14 | 242000 | 0.14 |
| | Purified | 0.78 | 0.22 | 0.70 | 0.08 | 0.14 | 0.035 | 0.053 | 0.67 | 21 | 219500 | 0.15 |

$F_G$ and $F_M$, fraction of alginate consisting of guluronic and mannuronic acid, respectively. $F_{GG}$ and $F_{GGG}$, fraction of alginate consisting of guluronic acid in blocks of dimers and trimers, respectively. $F_{MM}$, the fraction of alginate consisting of mannuronic acid dimers. $F_{MG}/F_{GM}$, fraction of alginate consisting of mixed sequences of guluronic and mannuronic acid. $F_{GGM}/F_{MGG}$, fraction of alginate which starts or ends with a block of guluronic acid. $F_{MGM}$, fraction of alginate consisting of two mannuronic acids interspaced with a guluronic acid. $N_{G>1}$, average length of guluronic acid blocks. MW, molecular weight.

($F_M$) in the alginate conversely decreased with the purification process, along with the proportion of all other blocks analyzed that contained mannuronate.

### 3.3.2 Transplantation of encapsulated islets

Islets were cultured for one day following isolation, encapsulated, then cultured for one day further. Islets were then transplanted into the peritoneal cavity of diabetic BALB/c recipients. Blood glucose concentrations in the transplanted animals returned to normal levels within 24 hours following transplantation. Animals were followed over time and removed from study when they exhibited two successive blood glucose readings greater than 17mmol/l. When these animals were followed over time, 90% of mice transplanted with the purified alginate showed graft survival greater than 100 days, with a mean survival time of 139 days (Table 3-2). This is compared to a survival rate of 69% and a mean survival times of 118 days among animals transplanted with non-purified alginate.

**Table 3-2.** Effect of alginate purification on the transplant survival of syngeneic BALB/c islets

| Alginate | n | Days of Euglycemia (MST±SE) | % Survival (>100d) |
|---|---|---|---|
| **Non-purified** | 39 | 21x2$^{\dagger}$, 42x6$^{\dagger}$, 63$^{\dagger}$, 63$^{\ddagger}$, 84$^{\dagger}$, 84$^{\ddagger}$, 105x10$^{*}$, 105$^{\dagger}$, 126$^{\dagger}$, 133$^{\ddagger}$, 154$^{\dagger}$, 161$^{\ddagger}$, 175x2$^{*}$, 182$^{*}$, 182x2$^{\dagger}$, 189x3$^{*}$, 196x2$^{*}$, 217$^{*}$, 231$^{*}$ (118 ± 10) | 69.23 |
| **Purified** | 42 | 14$^{\ddagger}$, 20$^{\S}$, 42$^{\ddagger}$, 63$^{\dagger}$, 105x9$^{*}$, 119$^{*}$, 126$^{\ddagger}$, 133x2$^{\dagger}$, 149x3$^{\dagger}$, 155x10$^{*}$, 175x2$^{*}$, 182$^{*}$, 185$^{\ddagger}$, 189x2$^{*}$, 189$^{\dagger}$, 191x2$^{*}$, 210$^{*}$, 231x2$^{*}$ (140 ± 8)$^{**}$ | 90.48$^{**}$ |

Euglycemia was defined as blood glucose values ≤ 8.7 mmol/l. MST, mean standard time. $^{*}$Euthanized while normal. $^{\dagger}$Euthanized while hyperglycemic. $^{\ddagger}$Died while normal. $^{\S}$Died while hyperglycemic.

$^{**}p<0.05$ vs. non-purified alginate

There were animals in each group that were euthanized while still normoglycemic. When we plotted our data to observe the metabolic state of our animals over time, we observed that that the average blood glucose concentration of mice receiving islets encapsulated in purified alginate was significantly lower than that of mice receiving non-purified alginate capsules (Figure 3-1). Furthermore, blood glucose values of mice receiving islets encapsulated in purified alginate were not significantly different than control values.

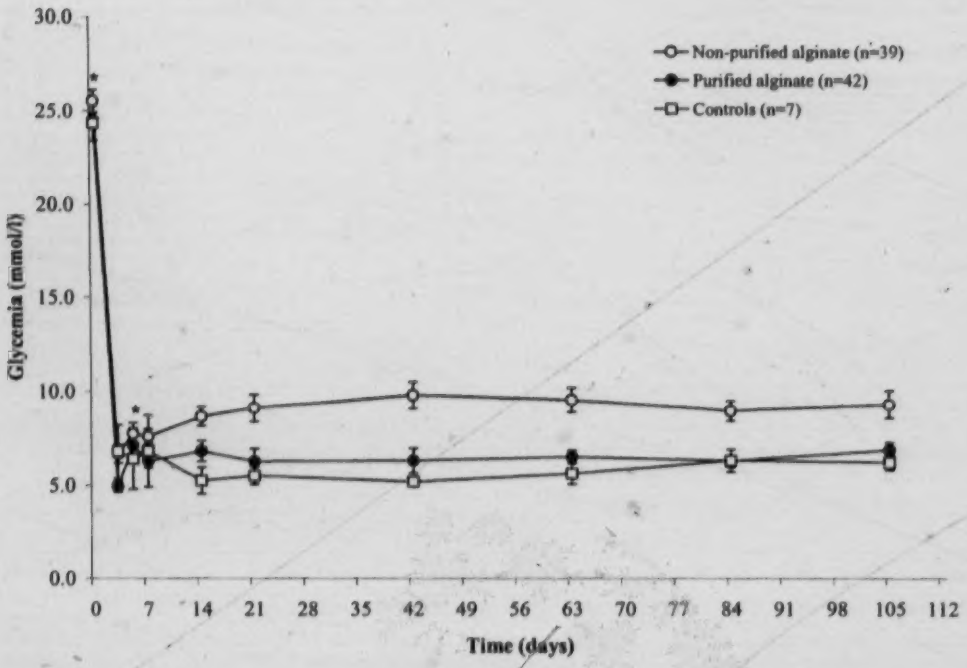


**Figure 3-1.** In vivo function of non-purified alginate encapsulated (○) or purified alginate encapsulated (●) BALB/c mouse islets transplanted into diabetic BALB/c mice, or controls (□). Data are expressed as means ± SEM ($p<0.05$). *$p<0.5$ vs. non-purified alginate.

### 3.3.3 Oral glucose tolerance test

An OGTT was performed on normoglycemic transplant recipients at approximately 105 days following transplantation (Figure 3-2). Animals transplanted with non-purified alginate encapsulated islets demonstrated significantly higher blood glucose concentrations throughout the OGTT than those receiving purified alginate. Furthermore, the AUC for the OGTT of recipients of non-purified alginate encapsulated islets was 1994.3±56.1min·mmol/l, significantly higher than the AUC of 1363.5±52.3min·mmol/l for those getting purified alginate ($p<0.05$).

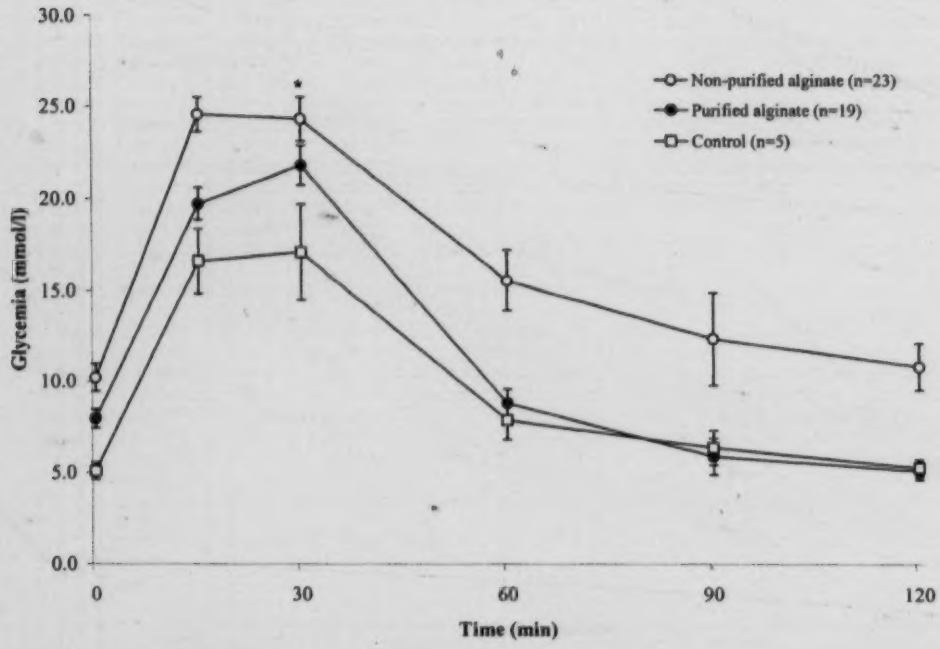


**Figure 3-2.** Oral glucose tolerance tests. Blood glucose profile for BALB/c mice with syngeneic transplants of i.p. non-purified alginate encapsulated islets (○), i.p. purified alginate encapsulated islets (●), or controls (□) after 105 days. Data are expressed as means ± SEM ($p<0.05$). *$p<0.2$ vs. non-purified alginate.

### 3.3.4 Recovery of microcapsules

Capsules were recovered at various time points greater than 100 days, at which time conditions were observed internally for graft recipients. Among mice receiving islets encapsulated in non-purified alginate, 12 of 27 (44.4%) animals showed signs of severe liver swelling, compared with 2 of 19 (10.5%) animals receiving islets in purified alginate (Table 3-3). When we observed the condition of capsules following recovery, animals transplanted with non-purified alginate encapsulated islets exhibited cellular overgrowth of the capsule in 20 of 27 cases (74.1%). Such cellular overgrowth was only evident in 3 of 19 cases (15.7%) when purified alginate was used (Table 3-3). Furthermore, when a sample was taken of non-purified alginate microcapsules which were overgrown, these capsules contained islets which were dark and which stained poorly with dithizone (Figure 3-3A). On the other hand, samples of non-overgrown, purified alginate microcapsules contained islets that were healthy looking and stained well with dithizone (Figure 3-3B).

**Table 3-3.** Effect of alginate purification on post-transplant recovery conditions and insulin secretory activity

| | | Conditions on Islet Recovery | | Insulin secretory activity | | |
|---|---|---|---|---|---|---|
| **Condition** | **n** | **Hepatic swelling (%)** | **Capsular overgrowth (%)** | **2.8 mmol/l glucose (%)** | **20 mmol/l glucose (%)** | **Stimulation indices High: low (%/%)** |
| **Non-purified** | 27 | 44.4 | 74.1 | 1.4 ± 0.2 | 5.2 ± 2.8 | 2.9 ± 1.1 |
| **Purified** | 19 | 10.5* | 15.7† | 1.1 ± 0.2 | 11.6 ± 3.5 | 7.9 ± 3.5* |

Data expressed as percentages or as means ± SE. *p<0.05, †p<0.001 vs. islets in non-purified alginate capsules.

### 3.3.5 Assessment of insulin secretion

Recovered capsules from each mouse were divided into two groups and cultured for 2 hours in the presence of either low or high glucose, assayed, and expressed as a percentage of the total insulin content. Encapsulated islets cultured in the presence of 2.8mmol/l glucose showed insulin release values of 1.4% and 1.1% from non-purified and purified alginate capsules, respectively (Table 3-3). On the other hand, when cultured in the presence of 20mmol/l glucose, insulin release values from encapsulated islets were 5.2% and 11.6% from non-purified and purified alginate capsules, respectively. As a result, non-purified alginate encapsulated islets demonstrated an

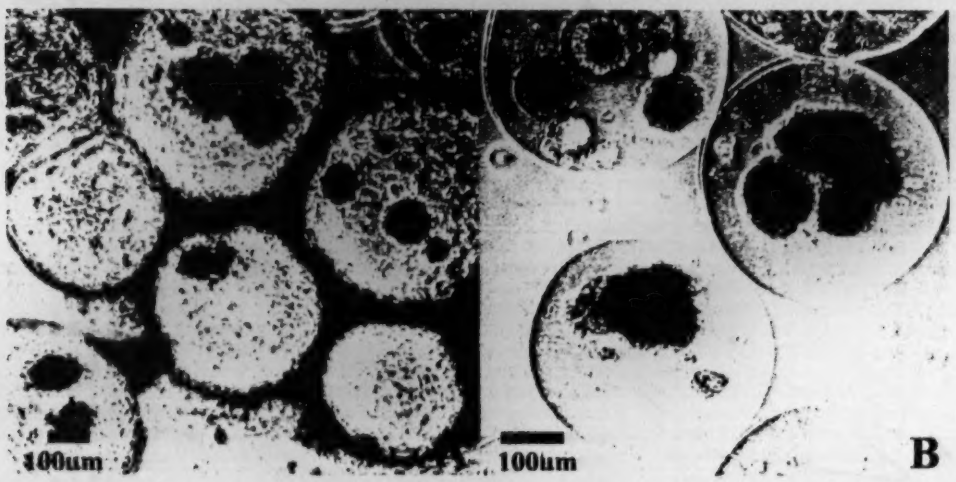


**Figure 3-3.** Representative light micrographs of dithizone-stained BALB/c islets encapsulated in non-purified alginate (A) or purified alginate (B) following implantation into and recovery from the peritoneal cavity of BALB/c mice. In the absence of alginate purification, transplanted islets demonstrated the presence of cellular overgrowth, and islets while staining peripherally for dithizone were often dark and necrotic in the center of the islet. Islets transplanted in purified alginate looked healthy and stained well with dithizone.

average stimulation index of 2.9 ± 1.1, while purified alginate encapsulated islets showed a value of 7.9 ± 3.5 (Table 3-3).

## 3.4 DISCUSSION

The purpose of this study was to determine whether purification of commercially available alginate would lead to an improvement in survival and function of encapsulated syngeneic islets transplanted into an immune competent mouse model. We have shown previously that encapsulation of islets leads to improved survival and metabolic function in long term tissue culture compared to non-encapsulated islets, and that these islets demonstrate outstanding function when transplanted into an immune-deficient recipient (20). With acceptable biocompatibility of the capsules in an immune compromised recipient, progression to an immune competent recipient was the next step, not only to assess the capsules for biocompatibility but also for their level of immune protection (21). Specifically, we wanted to analyze the transplantation of encapsulated islets into syngeneic recipients that we might study the effect of the host immunity on the capsule itself. Preliminary work has led us to believe that the commercially available sources of alginate were not ideal. This led our efforts to an exploration of methods to purify, alter or optimize the alginate for improved islet survival and function following transplantation. Other groups have similarly studied the effects of altering commercially available alginate through enzymatic modification (22) or purification (12;23) with varying results.

Alginate purification in our study led to some interesting observations. To begin with, we expected that ultra-filtration against an 8,000NMWL membrane and subsequent

removal of very short-chain alginate molecules would lead to an overall increase in the average molecular weight of the final alginate product. Such was not the case. The resultant decrease in the molecular weight of the purified alginate may have been due to degradation of large chains into smaller ones (24), or to selection for shorter versus longer chains at some other point of the purification. More likely, however, is that longer chains became trapped in and were not adequately recovered from the Centricon ultra-filtration devices. Although the literature seems to indicate that an alginate of a reduced average molecular size leads to a decrease in stability (25), our change was relatively small, and led to no apparent loss of stability in the purified capsules. Additionally, it has been recently proposed that removal of monomers and small oligomers of mannuronic and guluronic acid may be beneficial, in that their release from transplanted capsules *in vivo* may contribute to the immunogenicity of the capsule (26). Furthermore, we were initially surprised to see that endotoxin levels remained unchanged through the purification process, however this should not be surprising considering that endotoxin concentrations in commercially available purified alginates are already low (our blank control demonstrated a value of 0.18 IU/ml) and certainly under acceptable limits (13). Nonetheless, on the point of endotoxins it can be observed that the later second batch alginate demonstrated notably lower levels than the first batch, demonstrating the progress made by industry in reducing these levels.

The result perhaps most interesting regarding the alginate purification was the notable increase in the relative guluronic acid (G) content of the post-purification versus pre-purification alginate. It has been well documented that alginates of a higher the G-block concentration demonstrate greater stability (25). This is significant as the increase in

stability reflects a decrease in the likelihood of capsule breakage (27) resulting in a reduced incidence of direct exposure of our cells to the immune system. Furthermore, there have been proposed benefits to reducing the M-content of alginate capsules, particularly with regards to reducing the immunogenicity of the capsules (28;29). There have been varying opinions over the years as to the preferences of researchers for alginates that are classed as either G-rich (higher stability, lower porosity) or M-rich (lower stability, higher porosity). Klöck et al. indicate that the results of comparisons between alginates are more the result of the processes used to purify the alginate (30), thus making objective comparison between batches of alginate difficult. Whereas our results seem to indicate that improved graft function and longevity of survival were the result of the increased G-content of our purified alginate, it is certainly possible that our improved findings with purification are the result of the removal of other elements such as proteins or polyphenols (14;31) for which we have not tested.

Transplantation of islets into our recipients showed near immediate restoration of normal blood glucose values, irrespective of whether the islets were encapsulated in purified or non-purified alginate. Whereas blood glucose values were quite similar for the first week following transplantation, differences between the groups became pronounced and significant from 2 weeks post-transplant and followed consistently through to the end of the study. This data was strengthened by our large sample sizes (n=39 non-purified alginate capsule recipients; n=42 purified alginate capsule recipients). Furthermore, our OGTTs in mice transplanted with purified alginate capsules showed blood glucose values comparable to control animals at points from 60 to 120 minutes. The lag in response at 0, 15 and 30 minutes can easily be explained by the fact that the encapsulated islets never

develop direct vascular support, meaning that glucose must first diffuse through the capsule and into the center of the islets where the β-cells are located, and that insulin then must be released from the capsule and absorbed through the peritoneum, leading to lag (32;33). Mice transplanted with non-purified alginate were significantly higher at all points throughout the OGTT. Whereas all of these results could be explained by toxic or other effects of the alginate itself on the islets contained within the microcapsule, it is more likely that the effect is the result of immune or other forces external to the capsule leading to a loss of viable islet cells within the capsule. This is supported by observation of our explanted microcapsules. Mice receiving islets encapsulated in non-purified alginate showed a 4.4 times greater incidence of cellular overgrowth on the capsules on recovery than mice receiving purified alginate. We have observed and it has been shown by others that the cells adhering to the surface of the microcapsules are predominantly macrophages (17;34-36). We suspect these macrophages to be the predominant cause of the reduced function of the grafts in these recipients by way of a number of possible effects. For example, nutrient or oxygen deficiency within the capsule has been proposed as a potential reason for graft failure with even non-overgrown islets in immune compromised animals (9;37). As we are using a simple, open-pore capsule of a relatively small size (450μm diameter), we doubt capsule properties alone to be the lone contributing factor of these potential deficiencies, however given the 'coating' of highly metabolically active macrophages on the surface, it is entirely reasonable that the accumulation of macrophages lead to a relative deficiency of oxygen & nutrients in the local microenvironment. Furthermore, overgrowth cells would lead to the local increase in metabolically active waste products of both the macrophages and the islets. Further

support for this is derived through simple observation of the overgrown islets (Figure 3-3A) which stained poorly with dithizone and were dark and necrotic, particularly in the core of the islet, an indicator for nutrient deficiency (38). The presence of central necrosis in these mice would especially lead to a loss of beta cells, given that beta cells in mice seem to reside in the core of the islet (39). Potentially, this could be less problematic with human islets where the beta cells are dispersed throughout the islet. Islet failure may also occur in a more specific manner. Macrophages overgrown on the surface of the capsule may release damaging cytokines, nitric oxide, or reactive oxygen species causing the death and/or reduced function of the contained islets. Given the simple, open-pore nature of our capsule, these substances, along with any products of necrosis could pass freely back and forth across the capsule (40-42). We would expect that this reaction would be primarily mediated via innate immunity as our islets were not transplanted across strain or species but were syngeneic. The question then arises as to the insult causing such a reaction. More than likely, such a reaction is due to more than one factor, some of which may include: the biocompatibility of the alginate, the streptozotocin used to induce diabetes, friction or mechanical forces between the capsule and the liver and peritoneum, or of the surgical procedure with subsequent scaring and inflammation. It is interesting to note that in studies with empty capsules of both purified and non-purified alginate, no capsules of either alginate transplanted and recovered at 1 or 3 weeks (n=10 in each group at each time) showed any signs of overgrowth or immune reactivity. This indicates that the alginate itself is not the sole immunogen, and that the presences of the islets, streptozotocin, or other factors are necessary for full immune reactivity. Furthermore, some of the non-specific causes of islet failure and necrosis

could potentially lead to the release of antigens provoking an inflammatory response. It was interesting to note that the predominant organ affected in transplant recipients was the liver, which in some of the animals (44.4% of those receiving non-purified alginate encapsulated islets capsules and 11.1% of animals getting purified alginate) would lose its flat, lobular appearance and swell up to resemble a more spherical mass. In these instances, the capsules were often found clustered around the liver. This did not occur in every case, though it occurred with a 4 fold greater frequency in recipients of non-purified versus purified capsules. Combine this with the more than 4 fold greater frequency of overgrowth in non-purified versus purified capsules, we would suspect that the composition of the alginate is a predominant element in this equation.

When we compared the insulin responsiveness of encapsulated islets recovered from animals receiving purified versus non-purified alginate, results were similar to those of the *in vivo*. Whereas insulin release in the presence of low glucose was similar for both purified and non-purified alginate encapsulated islets, culture of purified capsules in a high glucose media yielded more than twice the insulin release of islets in non-purified capsules. Similarly to what was previously observed, reduced insulin responsiveness here can be explained by the loss of functional beta cells over time.

Our findings indicate that alginate purification leads to significantly improved survival and function in encapsulated islets transplanted into immune competent syngeneic recipients. Nevertheless, much work yet needs to be done. Graft failure, whether by non-specific causes (43), by microcapsule material elicitation of a damaging inflammatory response (44), or other factors, needs to be further analyzed in order to have a model suitable for human transplantation. Macrophage overgrowth is, by our

estimation, the greatest single factor leading to the failure of our transplants. Along this line of thinking, encapsulation along with macrophage depletion has been reviewed as a possible solution (17), though the effect of this treatment on the long-term survival of these islets *in vivo* is uncertain. Whether this macrophage mediated failure is the result of nutrient deprivation or specific factors leading to the death of islet cells, or both, is as yet not entirely clear. What is certain is the need to provide continued protection to the islets, particularly as we move toward effective protection in allogeneic and xenogeneic models. This is especially important in light of the fact that macrophages are extremely effective antigen presenting cells, and could potentially take-up and present shed antigens from the capsule and subsequently activate an even more vigorous innate response (45). Such a heightened innate response further may act as an adjuvant to a more problematical T-cell mediated adaptive response. In any case, it is unlikely that encapsulation will provide the entire solution to the problem of graft failure in the absence of immune suppression. Rather, it is more probable that this technology will be one part of a more complex solution.

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# CHAPTER 4

# TRANSPLANTATION OF CO-ENCAPSULATED OF NEONATAL PORCINE SERTOLI CELLS WITH ISLETS REDUCES IMMUNE RESPONSE BUT DOES NOT PROLONG ISLET FUNCTION

## 4.1 INTRODUCTION

It has been estimated there to be approximately 171 million cases of diabetes worldwide as of the year 2000, with incidence of the disease steadily growing (1). Islet transplantation has been offered as a possible cure for individuals affected with the disease; however, the chronic shortage of cadaveric donor tissue and the need for chronic immunosuppression has prevented this therapy from widespread use among the population of diabetic patients at large (2-5). More recently, xenotransplantation, particularly the use of islets isolated from pigs, has been proposed as a possible solution to the islet supply problem (6), with promising results in both lower primates (7;8) and human recipients (9). Furthermore, encapsulation of islets provides a means of mimicking the microenvironment the pancreas (10) and has been proposed as part of a possible solution to the problem of immune rejection by alleviating or eliminating the need for chronic immune suppression through isolation of the islets from host immunity (11-13). Additionally, Sertoli cells have been extolled for their ability to secrete factors suspected of having localized immune tolerizing properties (14-19). Taken together,

these principles may provide a means of making islet transplantation more readily accessible to the ever growing population of individuals with diabetes.

The purpose of this study was to examine the effects of transplanting co-encapsulated neonatal porcine islets (NPI) with neonatal porcine Sertoli cells (NPSC). Specifically, we wanted to determine the ability of the Sertoli cells to act as 'nurse cells' promoting the maturation and function of the islets, and their ability to provide an *in vivo* microenvironment of immune privilege within and around the capsule in immune compromised and immune competent animals.

## 4.2 RESEARCH DESIGNS AND METHODS

### 4.2.1 Animals

Landrace-Yorkshire crossbreed pigs (Swine Research and Technology Centre, University of Alberta) were used as donors of both islets and Sertoli cells. Donor pancreases were obtained from 1- to 3-day-old neonatal pigs (1.5–2.0 kg body wt) of either sex. Donor testes were obtained from male 1- to 3-day-old neonatal pigs (1.5–2.0 kg body wt). Male BALB/c mice aged 6-10 weeks (HSLAS, Edmonton, AB) and C57BL/6-rag1$^{tm1/mom}$ (B6 *rag1*$^{-/-}$, H2$^{b}$) (Jackson Laboratories, Bar Harbor, ME, USA) were used as recipients for transplantation. Diabetes was induced by intraperitoneal (i.p.) injection of streptozotocin (STZ; 275mg/kg body weight for BALB/c mice and 175mg/kg body weight for B6 *rag1*$^{-/-}$ mice, freshly dissolved in acetate buffer, pH4.5; Sigma Chemical Co., St. Louis, MO) 2 to 7 days prior to transplantation. Diabetes was confirmed by the presence of hyperglycemia (>18.0mmol/L). Blood was obtained from a snipped tail and blood

glucose levels were monitored with a portable glucose meter (One Touch Ultra, Johnson & Johnson, Milpitas, CA). All animals were maintained under conventional conditions in accordance with the recommendations of the Canadian Council on Animal Care.

### 4.2.2 Isolation and preparation of islets

The method used to isolate NPI has been previously described (6). Briefly, neonatal pigs were anesthetized under halothane and subjected to laparotomy and exsanguination. The pancreas was removed, placed in Hanks' balanced salt solution (HBSS) supplemented with 0.25% BSA (Sigma, St. Louis, MO), cut into 1- to 2-mm pieces, and digested with 1.0 mg/ml collagenase (Type V, Sigma). After filtration through a 450 μm nylon screen, the tissue was cultured in Ham's F10 medium (Life Technologies, Burlington, ON, Canada) containing 10 mmol/l glucose, 50 μmol/l isobutylmethlxanthine, 0.5% BSA, 2 mmol/l L-glutamine, 10 mmol/l nicotinamide, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C for 6 days with media changes every 2 days. At this time plates were recombined, and recombined to two tubes, one for encapsulation and subsequent culture and transplantation, and the other for co-culture with Sertoli cells for 48 hours prior to co-encapsulation.

### 4.2.3 Encapsulation of neonatal porcine islets

Following culture, aliquots of approximately 10,000 neonatal porcine islet equivalents (IE) were washed and re-suspended in 0.50 ml of Hank's balanced salt solution (HBSS) (Gibco) that was free of Ca++ and Mg++. They were then mixed with 0.50 ml of 1.5% (w/v) alginate purified in house as previously described (See Chapter 3) from Pronova UP-MVG alginate (high G alginate, batch #701-256-08; NovaMatrix, FMC Biopolymer,

Drammen, Norway) dissolved in HBSS (without Ca++, Mg++) (pH 7.4). Alginate microcapsules (400–500 μm in diameter) were formed using an electrostatic droplet generator (designed by Metabolex). Capsules were then cultured for 7 days in Hams F-10 media and 10% heat-inactivated neonatal porcine serum (NPS), supplemented as before.

### 4.2.4 Isolation and preparation of neonatal porcine Sertoli cells

Neonatal porcine Sertoli cells were isolated by collagenase and trypsin digestion as described previously (20). Sertoli cells were counted and cultured on non-tissue culture treated plastic Petri dishes (150x15mm, Fisher Scientific, Ottawa, ON, Canada) with neonatal porcine islets at a density ratio of $5x10^6$ Sertoli cells per 5,000 neonatal porcine IE per plate in supplemented Ham F10 and 10% heat-inactivated NPS. Islets and Sertoli cells were cultured 48 h at 37°C prior to encapsulation and subsequent transplantation.

### 4.2.5 Co-encapsulation of islets and Sertoli cells

Following culture, aliquots of approximately 10,000 neonatal porcine IE plus $10 \times 10^6$ Sertoli cells islets were washed and re-suspended in 0.50 ml of Hank's balanced salt solution (HBSS) (Gibco) that was free of Ca++ and Mg++. They were then mixed with 0.50 ml of 1.5% (w/v) alginate purified in house as previously described (See Chapter 3) from Pronova UP-MVG alginate (high G alginate, batch #701-256-08; NovaMatrix, FMC Biopolymer, Drammen, Norway) dissolved in HBSS (without Ca++, Mg++) (pH 7.4). Alginate microcapsules (400–500 μm in diameter) were formed using an electrostatic droplet generator (designed by Metabolex). Capsules were then cultured for 7 days in supplemented Hams F-10 media and 10% heat-inactivated NPS as before.

### 4.2.6 Transplantation and metabolic follow-up

After 7 days of culture in Hams F-10 and 10% NPS, co-encapsulated NPI with NPSC were washed twice in HBSS and suspended in sterile saline with 12mmol/l $CaCl_2$. Prior to implantation, in order to standardize the volume of neonatal porcine islets transplanted in each recipient, representative aliquots of each preparation were counted and sized to determine total islet equivalents. Graft mass varied between 2500 to 10000 neonatal porcine islet equivalents with 2.5-10 x $10^6$ Sertoli cells, respectively. Furthermore, other BALB/c mice and B6 *rag1*$^{-/-}$ mice were transplanted with encapsulated NPI alone. Capsules were then implanted into the peritoneal cavity with an 18 gauge cannula via a small incision in the *linea alba*. The incision was closed using MikRon Autoclip stainless steel wound clips (9mm; Becton Dickson, Sparks, MD). Mice were monitored over time for blood glucose levels between 8:00 and 11:00 a.m. When the blood glucose level was $\leq$8.7 mmol/l the graft was deemed a success.

### 4.2.7 Graft recovery

At various time points throughout the study, capsules were recovered by an intraperitoneal lavage with phosphate buffered saline containing 12 mmol/l $CaCl_2$. From the recovered grafts, a small sample was taken and stained with 0.02% dithizone (Sigma) in PBS to observe islet morphology. Recipient animals were assessed for the presence of non-specific peritonitis and swelling of the liver. Islets were observed also for the presence or absence of necrosis and for the general condition of the tissue. Capsule recovery was observed based on whether the capsules were free floating in the peritoneum or clustered and adhered to the liver or other internal organs. Capsules were also observed for shape, size and for the presence or absence of breakage and/or fibrotic

overgrowth. Furthermore, a sample of recovered capsules were scored individually and averaged on an arbitrary scale of 0 to 3 based on the amount of overgrowth present: 0 – no overgrowth observed; 1 – cells covering less than half the capsule one layer thick, 2 – cells covering more than half of the capsule one layer thick; 3 – cells covering the entire capsule 2 or more layers thick. To confirm the efficacy of the encapsulated islets at correcting diabetes, the pancreas of normalized recipients were assayed for insulin content as previously described (6).

Of the remaining recovered capsules, we assessed the insulin secretory responsiveness of the encapsulated islets during a 2 h static incubation at 37°C. The recovered encapsulated islets were washed twice in HBSS, then once in Hams F-10 culture media. The tissue was separated into two samples, each sample incubated in 1.5mL Hams F-10 supplemented with 2 mmol/L L-glutamine, 0.5% BSA and either 2.8 mmol/L glucose or 20 mmol/L glucose. Following the 2 hour incubation at 37°C the supernatant was collected and evaluated for insulin content by radioimmunoassay (RIA). The tissue was collected, washed twice for 10 minutes at 37°C with dissociation media to dissolve the capsules, following which the tissue was also assayed for total insulin content. The insulin content of the medium was expressed as a percentage of the total content (i.e., tissue plus medium). Stimulation indices were calculated by dividing the amount of insulin release at 20 mmol/l glucose by release at 2.8 mmol/l glucose.

### 4.2.9 Statistical analysis

Data are expressed as means ± SEM of *n* independent samples. Statistical significance of differences was calculated with a two-tailed unpaired Student's *t* test or one-way analysis of variance (ANOVA) for multiple comparisons. In the case of dichotomous data, a

Fisher's Exact Test (Chi-squared analysis) was used. Findings were deemed significant at a $p$ value of less than 0.05.

## 4.3 RESULTS

### 4.3.1 Transplantation of encapsulated islets

Following co-encapsulation and tissue culture of NPI with NPSC, capsules were transplanted into BALB/c mice and immune compromised B6rag-/- mice with diabetes induced by STZ injection. When we observed these mice over time, it became clear that following the first 3 weeks post-transplantation the B6 *rag1*$^{-/-}$ mice began to show a decrease in blood glucose levels, whereas the BALB/c mice showed a slight increase (Figure 4-1). At 18 weeks, this rise became more pronounced as BALB/c mice showed a more dramatic rise in blood glucose while B6 *rag1*$^{-/-}$ mice showed a dramatic reduction in blood glucose. This progression was steady and significant in each group, until 27 weeks when the BALB/c mice were terminated due to failure to gain weight, chronic hyperglycemia, and declining health, at which time capsules from these mice were recovered for testing. Furthermore, none of the BALB/c mice transplanted ever achieved normoglycemia (Table 4-1). On the other hand, B6 *rag1*$^{-/-}$ mice were allowed to continue to thrive, and by 34 weeks the average blood glucose concentration of these surviving mice finally dropped to normoglycemic levels. Of the B6 *rag1*$^{-/-}$ mice transplanted with NPI and NPSC, 11 of 24 (46%) showed normalization of their blood glucose concentration. The B6 *rag1*$^{-/-}$ mice were maintained for up to 48 weeks, following which these mice were euthanized and the capsules recovered.

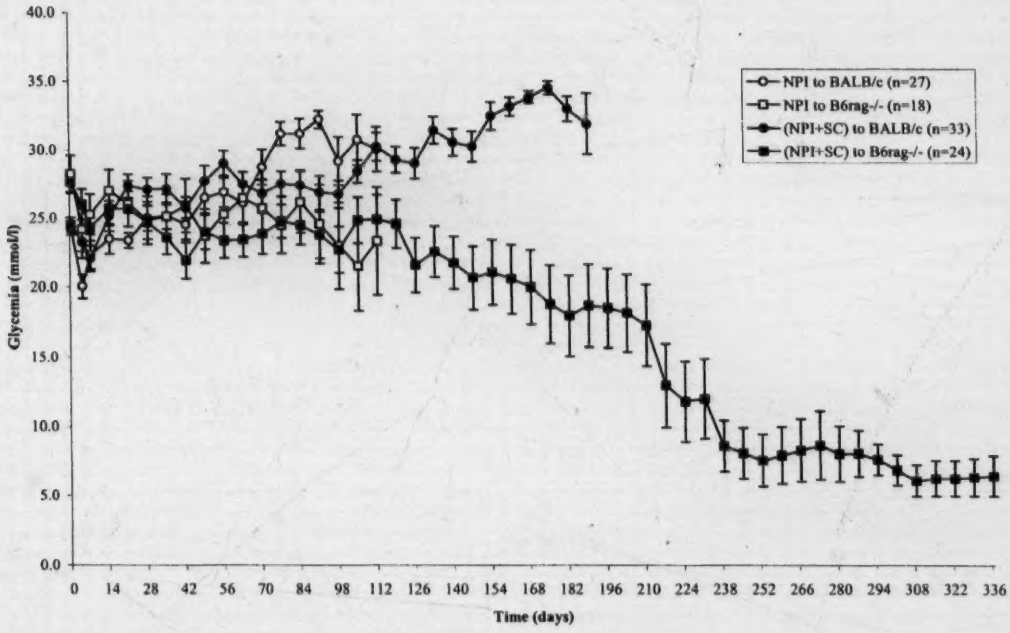


**Figure 4-1.** Average blood glucose concentrations of BALB/c mice transplanted with encapsulated neonatal porcine islets with (●) or without (○) co-encapsulated Sertoli cells, or of B6 *rag1*$^{-/-}$ mice transplanted with encapsulated neonatal porcine islets with (■) or without (□) co-encapsulated Sertoli cells, plotted over time. Data are expressed as means ± SEM.

When mice were transplanted with encapsulated NPI alone (i.e. no co-encapsulated NPSC), significance between blood glucose values in BALB/c mice and B6 *rag1*$^{-/-}$ mice was not observed until 10 weeks, when values rose for BALB/c mice and fell for B6 *rag1*$^{-/-}$ mice. When compared to mice receiving co-encapsulated NPI and NPSC, no discrepancy could be observed in the blood glucose of same strain mice receiving encapsulated NPI alone. Just as with BALB/c mice transplanted with

**Table 4-1** – Co-encapsulated islets and Sertoli cells transplanted into diabetic mice.

| Group | Islet mass (I.E.) | Sertoli mass (x $10^6$) | n | Days of survival (MST±SE) | % Achieving Normoglycemia |
|---|---|---|---|---|---|
| BALB/c | 5100 | 0 | 10 | 56 x 1*, 77 x 2†, 91 x 4†, 112 x 3† (91.0±5.7) | 0 |
| | 7800 | 0 | 9 | 4 x 1*, 28 x 1*, 56 x 1*, 77 x 3†, 91 x 2†, 112 x 1† (68.1±11.2)** | 0 |
| | 10000 | 0 | 8 | 14 x 1*, 42 x 1*, 63 x 3†, 147 x 3† (85.8±18.8) | 0 |
| B6 *rag1*$^{-/-}$ | 5100 | 0 | 5 | 77 x 2†, 91 x 1†, 112 x 2† (93.8±7.9) | 0 |
| | 7800 | 0 | 7 | 4 x 1*, 77 x 3†, 91 x 1†, 112 x 2† (78.6±13.8) | 0 |
| | 10000 | 0 | 6 | 63 x 2†, 77 x 1†, 168 x 1‡, 168 x 1†, 182 x 1‡ (120.2±23.7) | 33 |
| BALB/c | 2500 | 2.5 | 5 | 2 x 1*, 21 x 1*, 28 x 1*, 42 x 1*, 49 x 1* (28.4±8.2)** | 0 |
| | 3200 | 3.2 | 10 | 1 x 2*, 56 x 8† (45.0±7.3)** | 0 |
| | 5100 | 5.1 | 5 | 1 x 1*, 112 x 4† (89.8±22.2) | 0 |
| | 5800 | 5.8 | 5 | 91 x 1*, 182 x 1*, 189 x 3† (168.0±19.3)** | 0 |
| | 10000 | 10 | 8 | 14 x 1*, 112 x 1*, 147 x 3†, 175 x 3† (136.5±19.1) | 0 |
| B6 *rag1*$^{-/-}$ | 2500 | 2.5 | 5 | 210 x 1‡, 210 x 4† (210.0±0.0) | 20 |
| | 4700 | 4.7 | 9 | 280 x 1*, 336 x 1†, 336 x 7‡ (329.8±6.2)§§ | 89 |
| | 5100 | 5.1 | 3 | 112 x 3† (112.0±0.0) | 0 |
| | 5800 | 5.8 | 4 | 224 x 1*, 238 x 1‡, 238 x 2† (234.5±3.5)§§ | 25 |
| | 10000 | 10 | 3 | 168 x 1‡, 168 x 1†, 182 x 1† (172.7±4.7)§§ | 33 |

Euglycemia defined as blood glucose values ≤8.4 mmol/L. I.E., islet equivalents; NPSC, neonatal porcine Sertoli cells; MST, mean standard time. *Animal died while hyperglycemic, §Animal died when normoglycemic, †Grafts recovered from ***hyper***glycemic recipients for morphological assessment, ‡Grafts recovered from normoglycemic recipients for morphological assessment. **$p<0.05$, §§$p<0.01$ vs. animals receiving 5100 I.E. in each group.

co-encapsulated NPI and NPSC, none of the BALB/c mice transplanted with encapsulated NPI alone showed any normalization of blood glucose. On the other hand, of the 18 B6 *rag1*$^{-/-}$ mice transplanted with encapsulated NPI alone, 2 animals (11%) demonstrated a normalization of blood glucose, both of which received approximately 10,000 IE. When we observed the overall survival of mice of either stain receiving co-encapsulated NPI and NPSC or encapsulated NPI alone, only the B6 *rag1*$^{-/-}$ mice receiving co-encapsulated NPI and NPSC showed any significant difference from other groups, having a substantially longer survival time.

### 4.3.2 Recovery of microcapsules

Upon recovery of microcapsules from transplanted recipients, a number of factors were assessed. First, we looked for and recorded the presence or absence of peritonitis and liver swelling in recipient mice. Among grafts recovered from BALB/c mice transplanted with co-encapsulated NPI with NPSC, liver swelling was present in 28% (5/18) of the mice (Table 4-2). Of the mice transplanted with encapsulated NPI alone, 57% (12/21) demonstrated the presence of liver swelling. Among the B6 *rag1*$^{-/-}$ mice transplanted, neither those receiving co-encapsulated NPI and NPSC nor those receiving encapsulated NPI alone demonstrated any occurrence of liver swelling at the time of capsule recovery.

Next, we observed the state of the capsules on recovery, specifically whether we found capsules free floating individually in the peritoneum, or whether capsules were adhered together to the internal organs, or both. Of the 18 co-encapsulated NPI with NPSC grafts recovered from BALB/c mice, 15/18 (83%) had capsules free floating in the peritoneum, with 7/18 animals (39%) having capsules stuck to top of the liver

**Table 4-2** – Co-encapsulated islets and Sertoli cells recovered from transplanted mice

| Group | Islet mass (I.E.) | Sertoli mass (x $10^6$) | n | Liver swelling (%) | Capsule O/G (Avg. 0 - 3) | Free floating (%) | Clustering (%) | Insulin Secretion G-50 (%) | Insulin Secretion G-439 (%) | Static insulin release (Mean±SEM)(n) |
|---|---|---|---|---|---|---|---|---|---|---|
| **BALB/c** | 5100 | 0 | 9 | 44 | 1.63±0.15 | 33 | 89 | 1.17±0.46 | 2.41±0.27 | 3.42±1.17 (4)† |
| | 7800 | 0 | 6 | 50 | 1.50±0.12 | 50 | 67 | 1.49±0.53 | 2.06±0.82 | 1.20±0.27 (4)‡ |
| | 10000 | 0 | 6 | 83 | 1.83±0.19 | 50 | 67 | 0.07±0.01 | 0.34±0.11 | 4.86±0.61 (4)‡ |
| | AVERAGE | | 21 | 57 | 1.65±0.15 | 43 | 76 | 1.08±0.31 | 1.85±0.41 | 2.82±0.65 (12) |
| **B6 *rag1*$^{-/-}$** | 5100 | 0 | 5 | 0 | 1.75±0.27 | 100 | 20 | 0.25±0.07 | 2.19±0.80 | 14.98±6.82 (5)† |
| | 7800 | 0 | 6 | 0 | 0.88±0.12 | 100 | 33 | 0.11±0.03 | 0.97±0.17 | 12.20±2.69 (6)‡ |
| | 10000 | 0 | 6 | 0 | 0.30±0.04 | 50 | 33 | 0.12 | 0.37 | 3.05 (1)‡ |
| | AVERAGE | | 17 | 0†† | 0.93±0.14** | 82§§ | 29*** | 1.10±0.15 | 9.66±2.46 | 12.60±3.10 (12)§ |
| **BALB/c** | 3200 | 3.2 | 8 | 0 | 1.13±0.13 | 100 | 50 | 0.30±0.11 | 1.20±0.15 | 11.80±4.85 (8) |
| | 5100 | 5.1 | 4 | 25 | 0.27±0.05 | 100 | 25 | 0.09±0.02 | 1.26±0.27 | 16.05±4.39 (4)† |
| | 10000 | 10 | 6 | 50 | 0.65±0.06 | 50 | 33 | | | |
| | AVERAGE | | 18 | 28§§ | 0.78±0.09†† | 83§§ | 39§ | 0.68±0.28 | 7.33±3.24 | 13.21±3.48 (12)§ |
| **B6 *rag1*$^{-/-}$** | 2500 | 2.5 | 5 | 0 | 0.20±0.03 | 100 | 20 | | | |
| | 4700 | 4.7 | 7 | 0 | 0.57±0.02 | 100 | 0 | | | |
| | 5100 | 5.1 | 3 | 0 | 1.00±0.01 | 100 | 0 | 0.38±0.15 | 0.72±0.24 | 2.66±1.00 (3)† |
| | 5800 | 5.8 | 3 | 0 | 2.00±0.10 | 67 | 33 | | | |
| | 10000 | 10 | 3 | 0 | 0.00±0.00 | 100 | 0 | | | |
| | AVERAGE | | 21 | 0 | 0.67±0.03 | 95 | 10 | 0.38±0.15 | 0.72±0.24 | 2.66±1.00 (3) |

Liver swelling indicates the presence of peritonitis and swelling of the liver or other internal organs on recovery of the graft. Capsular overgrowth was assessed, given a number score and averaged within each group. NPSC, neonatal porcine Sertoli cells; I.E., islet equivalents; O/G, overgrowth; Clustering, presence of capsules adheres to top of the liver or other organs; MST, mean standard time. Static insulin release was performed at *56 days ‡77 days or †112 days post transplantation. §p<0.05, **p<0.002, ††p<0.001, §§p<0.02, ***p<0.01 vs. BALB/c transplanted with encapsulated NPI alone (no NPSC).

(Table 4-2). Among BALB/c mice receiving encapsulated NPI alone, 43% (9/21) had the capsules free floating, while 76% (16/21) animals showed capsules stuck to the liver. In the 21 NPI with NPSC grafts recovered from B6 *rag1*$^{-/-}$ mice, we found 20 mice (95%) to have capsules free floating, with 2 mice (10%) observed with capsules stuck to the top of the liver. B6 *rag1*$^{-/-}$ mice receiving only encapsulated islets showed 82% (14/17) with capsules free floating, and 29% (5/17) with capsules adhered to the internal organs.

Next, we assessed the recovered capsules for the presence and amount of cellular overgrowth on the capsules. Observing recovered samples of the capsules under the microscope, we scored individual capsules and averaged the scores on a scale of 0-3. BALB/c mice transplanted with co-encapsulated NPI and NPSC demonstrated an overall average score of 0.78±0.09, significantly less than the BALB/c mice transplanted with encapsulated NPI alone that showed an average overgrowth score of 1.65±0.15 (Table 4-2). The average score of 0.67±0.03 among B6 *rag1*$^{-/-}$ mice receiving NPI and NPSC capsules was also significantly lower than that of the capsules recovered from the B6 *rag1*$^{-/-}$ mice transplanted with encapsulated NPI alone, which showed an overgrowth score of 0.93±0.14 for the capsules.

Finally, we observed the gross morphology of our recovered NPI in the presence of dithizone staining from animals both with and without co-encapsulated with NPSC. Representative samples of capsules with NPI alone recovered from BALB/c mice exhibited maintenance of capsule shape, size and integrity. Islets stained well with dithizone, though many of the islets were dark, particularly in the center of the islet, indicating the presence of central necrosis. The presence of cellular overgrowth was evident. Samples of capsules recovered from BALB/c mice receiving both NPI and

NPSC, and from B6 *rag1*$^{-/-}$ mice (NPI with or without NPSC) revealed that capsule shape and integrity were consistent with pre-transplantation capsules, though the size of the capsules may have been diminished somewhat. Islets recovered from these animals looked healthy and stained well with dithizone, and showed a general absence of central necrosis. The incidence of cellular overgrowth in these three groups of animals was noticeably less than in NPI alone capsules recovered from BALB/c mice.

### 4.3.4 Assessment of insulin secretion

When we assessed the static insulin release of our recovered capsules, we noted that the average stimulation index for NPI co-encapsulated with NPSC recovered from BALB/c recipients was 13.2±3.5 (Table 4-2). This was made up of a one group where capsules were recovered at 56 days (average SI = 11.8±4.9) and another group where capsules were recovered at 112 days (16.1±4.4). BALB/c recipients receiving encapsulated NPI alone showed significantly diminished stimulation index of 2.82±0.65 among capsules recovered at 77 (1.20±0.27 and 4.86±0.61) or 112 (3.42±1.17) days. The average stimulation index for NPI co-encapsulated with NPSC recovered from B6 *rag1*$^{-/-}$ mice, all recovered at 112 days, was 2.7±1.0. Samples were also collected from one other group of B6 *rag1*$^{-/-}$ mice at 336 days, though the data was not included for the purposes of this study due to the presence of bacterial contamination in the culture medium. B6 *rag1*$^{-/-}$ mice transplanted with encapsulated NPI alone demonstrated substantially elevated average stimulation index of 12.60±3.10 among capsules recovered at the same time points (Table 4-2).

## 4.4 DISCUSSION

Transplantation of capsules containing NPI and NPSC into immune compromised B6 *rag1*$^{-/-}$ mice yielded some expected results. Specifically, immune compromised mice showed a slow and steady decline in average blood glucose concentrations, eventually leading to normoglycemic levels. Notwithstanding, blood glucose did not drop as quickly as we would have expected, nor did as many B6 *rag1*$^{-/-}$ mice become normoglycemic as we would have expected, particularly in light of past results we have achieved when transplanting xenogeneic tissue into immune compromised BALB/c mice (21). Furthermore, we were disappointed that none of our immune competent BALB/c mice transplanted with neonatal pig islets, with or without Sertoli cells, ever became euglycemic. In fact the average blood glucose concentrations of both groups of these animals increased over the course of the study. We would expect these results to be due to one or more of a number of factors, such as death of the islets, failure of the NPI to mature *in vivo*, or loss of function of the islets. The causes of these factors are not totally certain, though host immunity (including innate mechanisms in both strains of mice) is a likely candidate. Furthermore, even though the dose of NPI with NPSC varied among animals (from 2,500 NPI and $2.5 \times 10^6$ NPSC to 10,000 NPI and $10.0 \times 10^6$ NPSC per mouse), the concentration of islets and Sertoli cells within the microcapsules remained the same in each instance ($10 \times 10^6$ NPSC per 10,000 NPI per milliliter of 0.75% alginate). As such, it is possible that the concentration of NPSC co-encapsulated with NPI within the capsule was not optimal for the growth, development and function of the islets when these capsules were transplanted into mice. For example, given the high metabolic activity of Sertoli cells, it is not unlikely that, transplanted in such close

proximity to the islets and in too large numbers, the Sertoli cells could deplete the capsule microenvironment of oxygen and nutrients sufficient to effectively starve the islets. In such an instance, a lower concentration of Sertoli cells in relation to the number of islets may be beneficial. Comparisons of same species animals seemed to indicate that the presence of co-encapsulated Sertoli cells provided little or no benefit or detriment to the grafts with respect to blood glucose over time. Still, based on the limited analysis here, the precise effects of the Sertoli cells was difficult to determine. It was true that 46% of B6 *rag1*$^{-/-}$ mice transplanted with co-encapsulated NPI and NPSC achieved euglycemia over the course of the study, versus 11% of the control B6 *rag1*$^{-/-}$ mice receiving encapsulated islets alone. Though such results seemed to indicate that the presence of the Sertoli cells provided some level of benefit to these islets in the immune deficient state, arguably these results may have been the same had the B6 *rag1*$^{-/-}$ mice receiving encapsulated islets alone demonstrated health sufficient to continue past 112 days. Furthermore, it would seem as though there would be a balance to be found with respect to the immune-altering benefits of the Sertoli cells versus their potential nutrient-robbing effects.

Recovery of microcapsules from transplanted animals provided further insights. To begin with, the incidence of liver swelling in BALB/c mice was notably less in animals receiving co-encapsulated NPI and SC than in animals receiving encapsulated islets alone. This seems to indicate that despite what may be going on inside the capsule that the Sertoli cells provide anti-inflammatory support surrounding the outside of the capsule. Such anti-inflammatory properties of Sertoli cells have been well documented (22;23). Additionally, this anti-inflammatory effect was inversely related to the

transplant volume, with the animals that received larger volumes of transplanted cells demonstrating reduced anti-inflammatory potential than animals transplanted with smaller volumes (Table 4-2). Arguably, the anti-inflammatory properties of the Sertoli cells were not improved with increased numbers of Sertoli cells, at least not sufficient to handle the increased immunologic load brought about by the increased tissue transplant volume. The livers of B6 *rag1*$^{-/-}$ mice showed no indication of swelling in any recipients on recovery, despite having intact innate immunity. It could be surmised that the absence of T-cells to perpetuate the innate response allowed for this success (24).

On recovery, we observed capsules as free-floating in the peritoneum and/or existing as clusters adhered to the internal organs, particularly the liver. In both BALB/c and B6 *rag1*$^{-/-}$ mice, the presence of Sertoli cell in the capsule led to a substantial increase in the proportion animals with islets found free floating in the peritoneum (Table 4-2). Similarly, both strains of mice were shown to exhibit a dramatic decrease in the number of animals having capsules adhered to the liver and peritoneal organs when Sertoli cells were present (Table 4-2). Also, B6 *rag1*$^{-/-}$ mice showed a higher proportion of capsules free floating and a lower proportion stuck to the liver than did the BALB/c mice, both in the presence and absence of Sertoli cells, indicating the added benefit of the immune compromised environment. When we observed capsules under the microscope, capsular overgrowth in mice with co-encapsulated NPI and NPSC was significantly reduced when compared with mice receiving encapsulated NPI alone. Furthermore, we found that the amount overgrowth related directly to the state of the capsules on recovery, with a greater incidence of overgrowth among capsules that were adhered together or to the liver, and a lesser incidence among mice where the capsules were predominantly free-floating (Table

4-2). This is not unusual, as we would expect it to be the overgrown cells that are actually the cause of capsule adherence to organs or to one another. As with liver swelling, capsular overgrowth was notably less in each condition in the B6 *rag1*$^{-/-}$ mice versus the BALB/c mice. It has been shown elsewhere that the cells predominantly involved in the cellular overgrowth of alginate microcapsules to be macrophages along with some CD4+ T-cells (25-27), and that the ablation of macrophages leads to a reduction in peritoneal inflammation (28). Furthermore, it has been shown that the depletion of peritoneal macrophages leads to improved xenograft survival in rodents (29;30). Taken together, all of these results indicate that there is significant benefit to both co-encapsulation of islets with Sertoli cells and to the immune compromised state, at least microscopically, with respect to the immune response of the host to the transplanted encapsulated islet. Keeping all of this in mind, we suspect that the Sertoli cells must be releasing factors that are in some way inhibitory to macrophage activation or function, or at least inhibitory to the adherence of macrophages to the microcapsule. While it has been shown that Sertoli cells have the ability to regulate the function of testicular macrophages (31;32), how this may occur outside of the testes with peritoneal macrophages has not been investigated. Finally, with the incidences of peritonitis, liver swelling, adherence to the liver and cellular overgrowth demonstrably less among immune compromised B6 *rag1*$^{-/-}$ mice than among immune competent BALB/c mice, the need for immune inhibition or some immune barrier beyond what the capsule alone can provide is well illustrated.

The presence of macrophages surrounding the capsule has generally been associated with a reduction in function of the islets both inside the capsule (26), as well

as impairing the function of islets in non-overgrown capsules in close proximity to the overgrown capsules (33). Since the microcapsule prevents direct cell-cell contact, we suspect that macrophage activation occurs in response to antigens shed across the capsule membrane. These antigens may be proteins or factors produced and released from the capsule by islets such as VEGF (34) or MCP-1 (35), which have been shown to be released by islets lacking direct vascular access. It is likely also that cellular contents released as cells within the capsule die initiate an inflammatory response and macrophage activation. It was our hope with these experiments that the accompaniment of the Sertoli cells within the capsule would provide a level of protection from the effects of this macrophage overgrowth. While it certainly was the case that Sertoli cell presence within the capsule correlated with a reduction in overgrowth and liver swelling, it is unfortunate that this did not especially lead to an increase in the normalization of blood glucose in immune competent BALB/c recipients. Furthermore, whether the reduction of swelling and overgrowth was the result of released Sertoli cell factors acting directly on the macrophages or T-cells, or indirectly through the prevention of CD4+ mediated up-regulation of the response is not totally clear. For example, Sertoli cells release TGF-β which is known to suppress T-cells (36). Nonetheless, the fact that a number of the B6 *rag1*$^{-/-}$ mice, known to lack functional B- and T-cells, showed normalization of blood glucose and a greatly reduced incidence of swelling and cellular overgrowth compared to BALB/c recipients would seem to indicate that further T-cell suppression would be key in the successful adaptation of this therapy (37).

When recovered islets were tested for their glucose stimulated insulin release in the presence of low and high glucose, we observed that BALB/c mice receiving co-

encapsulated NPI and NPSC demonstrated a substantially elevated stimulation index when compared to BALB/c mice transplanted with NPI alone (Table 4-2). The low SI exhibited by the NPI alone capsules recovered from BALB/c mice was a result of low insulin release from islets in the presence of high glucose, indicating a loss in islet function. It would seem that among BALB/c mice, the presence of Sertoli cells within the capsule served to increase or at least better maintain the islets' ability to respond to changes in blood glucose. On the other hand, when the same transplant conditions were performed in B6 *rag1*$^{-/-}$ mice, recovered capsules containing NPI and NPSC showed a considerably lower stimulated responsiveness to glucose than capsules containing only islets, which again was a result of a dramatic drop in insulin release at high glucose (Table 4-2). The reason for this discrepancy between BALB/c mice and B6 *rag1*$^{-/-}$ mice is uncertain, however the story here seems to be that the presence of Sertoli cells in the capsule is beneficial to islet function, and that the immune compromised state provided by the B6 *rag1*$^{-/-}$ mice is beneficial to islet response, but the cumulative effect of both is not of particular benefit, at least in this instance. Given the fact that the basal response to low glucose in each of the four groups is relatively constant, and that changes in the stimulated response are due to variations in the high glucose release of insulin, we would surmise that conditions, be they the transplant host or the microenvironment created by the presence or absence of NPSC within the capsule, are in some manner affecting islet responsiveness. Nonetheless, the reasons for this reduction in islet function elude us. In particular, why such a reduction in function should occur B6 *rag1*$^{-/-}$ is a mystery, short of these mice having incomplete or 'leaky' ablation of B- and T-cells.

It seems clear from this study that factors released from neonatal porcine Sertoli cells inhibit the immune and inflammatory responses to transplanted microcapsules containing these islets and Sertoli cells, though the ability of this state to translate into an improved condition for host recipients is of questionable value. Clearly the next step is to better identify the reasons for this, and how we might minimize any detrimental effects while harnessing the beneficial effects of the capsule and the Sertoli cell (38-40). While it is uncertain whether co-encapsulation with Sertoli cells will ever alone provide enough protection for islets to survive in immune competent recipients, we feel confident that further refinements to the procedure may have the potential to becoming one part of a safer, more viable therapy for the treatment of diabetes.

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# CHAPTER 5

# GENERAL DISCUSSION AND CONCLUSIONS

## 5.1 GENERAL DISCUSSION

Current estimates by the World Health Organization place the number of individuals with diabetes in 2000 at 177 million worldwide, with a more than doubling of the incidence of this condition projected by 2030 (1). Type 1 diabetes, the selective autoimmune destruction of β-cells in the islets of Langerhans of the pancreas, accounts for approximately 10% of these cases and is characterized by elevated blood glucose, along with a number of secondary microvascular and macrovascular effects. The costs of treating the disease are enormous, reaching into the billions of dollars in North America alone. Perhaps more significant that the financial burden of the disease, however, are the costs to patients and families related to the increased morbidity and mortality associated with the disease. Though lifelong, intensive insulin therapy has proved effective in preventing fluctuations in blood glucose and in reducing the chances of secondary complications, there remains with this therapy an increased risk of hypoglycemia episodes which can rapidly lead to coma and death if not treated. As a result, the transplantation of insulin-producing tissue remains the most promising treatment for type 1 diabetes.

Almost thirty years passed from the first successful islet transplantation of Ballinger and Lacy in rats (2) to the time that the group at Edmonton developed a process for consistently transplanting islets into type 1 diabetic patients (3), alleviating the need for exogenous insulin therapy. While the Edmonton protocol has moved islet

transplantation into the forefront of the attention of diabetes research and has provided hope to countless individuals affected by the disease, the procedure is still limited by two major obstacles that must be addressed in order for this therapy to become the standard of care for the entire population of patients with diabetes. One of these is the severe shortage of islets available for transplantation. When we consider that only 65 patients had undergone this treatment in the five years following the publication of the Edmonton protocol (4), and that the number of new diabetics in Canada alone numbers approximately 60,000 per year and growing (5), it is evident that we are far, far away from providing treatment that is universal in its accessibility under the current protocol. As a result of this lack of cadaveric donor islets, researchers have focused a great deal of attention on the search for alternative sources of insulin-producing, glucose responsive tissue for transplantation into diabetic recipients. One such reserve lies in the use of cells genetically engineered to secrete insulin (6-9), though these sources have in the past been limited by their ability to respond to the normal physiologic changes in blood glucose (6;10). Also among these potential sources are pancreatic progenitor cells (11-13), the use of which may well allow researchers to increase the pool of transplantable insulin-producing tissue obtainable from each pancreas. Unfortunately, much work remains to be done to understand the mechanisms and triggers involved in the identification of suitable progenitors and their subsequent differentiation into suitable endocrine tissue. Similarly, much investigation is underway into the generation of islets from embryonic stem cells (14;15). It is believed that within these cells lies the potential to generate a near limitless supply of insulin producing tissue. Still, this field suffers from a lack of understanding into many of the cues involved in forming pancreatic-like endocrine tissue,

and into the processes involved in switching on and off replication and differentiation. Furthermore, ethical controversy regarding the use of this tissue, particularly embryonic stem cell tissue, may hinder this work from moving forward in many arenas.

Perhaps most appealing among the alternatives to cadaveric human islet is the potential for xenogeneic sources of islets. While a number of these animal sources have been considered (16-18), perhaps the most promising of these are neonatal pigs. Pigs have the advantage of being easily housed and bred in large numbers, and islets from the neonates have been shown to be easily isolated in large numbers (18). Neonatal islet tissue has been shown to be effective in alleviating hyperglycemia in rodents (18;19), dogs (20), monkeys (21) and humans (22). Unfortunately, xenotransplantation is not without barriers to be overcome. The potential for cross-species disease transmission must be addressed and put to rest. Furthermore, as with embryonic stem cells, the use of xenogeneic tissue for human therapy has been a matter of public controversy. Finally, the risk of immune rejection must be addressed (as it must with all transplanted tissues), attention being particularly paid to the xenogeneic antigens for which there may exist a preformed response.

Clearly, prevention of rejection is an issue key to the future success of islet transplantation, and is the other major limitation of current clinical islet transplantation protocols. Under the Edmonton protocol, islet transplant recipients are required to trade in their insulin needles for aggressive chronic immunosuppressive drugs which, while prolonging the survival of the graft, carry a host of secondary complications. For many patients, particularly children with type 1 diabetes, the risks associated with these immunosuppressives far outweigh the risks associated with the disease. Furthermore,

recent evidence indicates that the likelihood of long-term survival of the islet transplant grafts even in the presence of immunosuppression is low, with only 10% of transplant recipients retaining insulin independence in a 5 year follow-up study (4). As such, researchers have been led to explore strategies to avoid immunosuppression. Efforts to reduce the immunogenicity of potential donor tissue – such as through the creation of transgenic animals – hold promise, though such animals only address one part of a much more complicated immunological problem. Other strategies have focused on the induction of immunologic tolerance of host recipients to donor tissues – the Holy Grail of transplantation – and while much is yet to be learned, understanding of the mechanisms involved in tolerance induction is growing. Immunoisolation of islets, particularly the use of alginate for islet encapsulation, is also an area of much interest, and has shown some success in the treatment of diabetes in both small and large animals. Furthermore, the use of nurse cells, like Sertoli cells have been shown to provide transplant support and relief from host immunity in a number of models.

It would seem as though with so many options and areas of research under considered in the realm of islet transplantation that success would be almost certain. Unfortunately, successes have been variable and, at times, fleeting. In particular, with the encapsulation of islets for transplantation, much of the research in this area has been largely outcome driven, with a lack of continuity between animal models of increasing immunity and with the failure of alginate capsule performance and immune isolation strategies left largely undefined. Our hope with this thesis was to explore these concerns as we moved from tissue culture to recipient, from immune compromised to immune

competent, from non-pure to purified alginate, and from simple capsule design to the addition of immunomodulatory Sertoli cells.

For the purposes of this work, the obvious place to start was the beginning. As a result, the choice was made to determine the effects of the capsule on the islets in the absence of active immunity. To start, survival and function of encapsulated islets were tested at various time points over a three week tissue culture period and compared to non-encapsulated islets at the same time points and under the same conditions. Under these conditions we were able to demonstrate (Chapter 2) the beneficial effects of the capsule on islet survival, as recovery at all time points was significantly improved versus non-encapsulated islets, and on islet function, with time points greater than one week showing superior function. While the reasons for these improvements are not totally certain, it can be elucidated that the abilities of the capsule to mimic the pancreatic matrix, maintain the integrity of the islet, and prevent aggregation of the islets in tissue culture all may be contributing factors. Continuing with this experiment we transplanted these recovered islets and demonstrated the capsules' ability to dramatically improve islet survival and function *in vivo* in an immune compromised host. One of the concerns presented with the use of encapsulated islet technology has been that the contained islet would not be able to function by diffusion alone, and in the absence of direct vascular access. We have here demonstrated that islet function can, in fact, be maintained in the peritoneum, at least in small animals. In fact, mice in these experiments transplanted with encapsulated dog islets demonstrated more rapid glucose clearance than even the age matched control mice (likely a result of a lower set-point for glucose of dog versus mouse islets). Regardless, animals receiving encapsulated islet transplants exhibited an ability to maintain

physiologic blood glucose levels over a substantial amount of time. Also, when we performed transplants of varying islet numbers in this series of experiments, we were able to demonstrate that the encapsulated islets not only had a superior ability to normalize blood glucose in recipients, but they could do so with dramatically reduced numbers of islets – consistently and with as few as 500 islets – as compared to non-encapsulated islets. This may have to do with the ability of the capsule to more accurately mimic the structural support matrix of the native pancreas, as mentioned before, or it may be a result of ability of the capsule to protect the islet from some of the potential shear and mechanical forces involved in the transplant process, leading to improved survival and overall function of the encapsulated islets post-transplant. Taken together, these experiments clearly demonstrated that islet encapsulation promotes the survival and function of islets both *in vitro* and *in vivo*, and that this technology warrants further investigation.

With benefit shown for islet encapsulation in the absence of active immunity, the goal then was to move to a model that allowed us to test the alginate in the presence of a functional immune system in order to determine any immune response to the capsular material in the context of encapsulated, non-immunogenic, functioning cells. Thus, we reviewed the use of alginate capsules in BALB/c mice receiving transplants of syngeneic encapsulated islets. Whereas empty capsules failed to elicit a response, initial experiments indicated that encapsulated islets did often induce active immunity against the graft. As these syngeneic graft failures occurred concurrent with a change in alginate supplier, this led also to an extensive review of the available literature on alginate purification and modification. The result was the development of a protocol for the

purification of alginate, which subsequently led us to the investigations of Chapter 3 of this work. In these experiments, we were able to analyze and specify a number of the changes that occurred with modification process. Furthermore, and more importantly, we clearly showed the result of this modification on the transplant success of islets encapsulated with this modified alginate. Recently, Dusseault et al. performed a comparative analysis of alginates purified by three different techniques (23). While such information is of interest, it is of little clinical consequence in the absence of transplantation data. While a comparison transplantation results using alginates of different sources has been approached elsewhere (24), what we have approached here is the first comparison of which we are aware on the effects of pre- and post-purification alginate on transplantation success using a simple alginate capsule. Our experiments have clearly shown that the purification process undergone here lead to prolonged survival and euglycemia, lower basal glucose maintenance, and improved insulin responsiveness to glucose. Furthermore, when capsules constructed from purified alginate were recovered from recipients there was a demonstrably reduced inflammatory presence as compared to non-purified alginate capsule recipients. Taken together, we are able to say with confidence what has always been suspected: that the quality and composition of any given alginate directly influences the longevity and performance of the encapsulated islet transplant.

The next logical step of this investigation into the viability of islet encapsulation and transplantation leads to a model of increased immune challenge. Whereas transplantation of encapsulated syngeneic islets allowed the specific study of host reaction to the capsular material itself, the transplantation of encapsulated donor islets

into immune competent non-syngeneic recipients allowed us to determine the level of immune protection afforded to the islets by the capsule. The study of allogeneic and xenogeneic islet transplantation provides us with markers of success or failure indicative of what might happen in the clinical realm. On this point, it is important to recognize that although we talk about encapsulation as 'immunoisolation', it is in fact primarily islet *cell* isolation, and not antigen isolation, per se. That is, the capsule has the ability to prevent cell-cell contact and thus direct *antigen* recognition of the islets. Nonetheless, the capsules are porous and will allow the passage of shed islet antigens from the capsule, allowing for indirect antigen recognition. Much discussion has ensued on this point with regards to the modification of capsule porosity as a means of controlling the amount of shed antigen presented to host immunity, and as a means of inhibiting the passage of antibodies, complement, cytokines and other immune effector molecules into the capsule (reviewed in (25)). It is important to remember here, however, that constricting the porosity of the capsule too aggressively also prevents the passage of therapeutic molecules, essential proteins, hormones, metabolic wastes, etc. into and out of the capsule. Certainly, a balance must be found, though a determination of what that cut-off porosity will undoubtedly be a matter for further discussion.

With these points in mind, Chapter 4 explores the ability of the microcapsules to protect donor islets from immune rejection. Xenograft transplantation was favored here simply for the ease of isolation of large numbers of (pig) islets for transplantation. In this chapter we transplanted neonatal pig islets and support previous work demonstrating that immune compromised mice receiving encapsulated islets had improved survival and normalization of blood glucose than immune competent mice. This supports literature

indicating that xenograft rejection occurs presumably through indirect CD4+ T cell recognition (26-29). This was further demonstrated through the recovery of grafts, which demonstrated capsules showing less evidence of immune challenge and islets of improved function in immune compromised over immune competent animals. Still, the ability of the islets to normalize blood glucose in these animals was exceedingly suboptimal, for which reason these experiments also explored the outcomes of co-encapsulation of neonatal pig Sertoli cells with the islets, transplanted again into immune compromised and immune competent animals. It has previously been shown that Sertoli cells transplanted under the kidney capsule increase the longevity of islet grafts in rats in the absence of immune suppression (30-32;32), thus demonstrating the potential benefit of Sertoli cells in circumventing exogenous immunosuppression. Here we demonstrated the ability of the Sertoli cells co-encapsulated with the islets to extend recipient survival, improve recovered islet function and exhibit reduced cellular overgrowth of recovered capsules in immune competent mice. Capsules containing islets and Sertoli cells retrieved from immune deficient mice demonstrated a similar reduction in host response to the capsule. Still, though clearly the presence of Sertoli cells had some benefit in subduing the immune response to the encapsulated islets, overt benefit to blood glucose levels in recipients were still not optimal, illustrating the need for further investigation into the specific factors released from the Sertoli cell to bring about these responses.

## 5.2 CONCLUSIONS AND FUTURE CONSIDERATIONS

Successes rates in clinical islet transplantation have dramatically improved since the inception of the Edmonton protocol. As a result, there has been a renewed interest into the field and a renewed hope for treatment of patients affected by type 1 diabetes. However, it is evident that current protocols fail to answer the problem of making this therapy widely available to the ever growing pool of individuals with the disease. As a result, alternative sources of tissue are being explored. Xenogeneic porcine islets are an attractive alternative to cadaveric human islets. However, for xenogeneic tissue to move to the forefront of clinical exploration, methods need to be developed that allow the transplantation and maintenance of healthy donor islets in recipients. Encapsulation of islets in alginate has demonstrated numerous advantages in this realm. Encapsulation allows for the maturation and prolongation of function of islets in tissue culture, and has shown to maximize the ratio of transplant dose to effect. Key to the success of this therapy is the quality and content of the alginate used, with alginate purification and modification protocols offering the opportunity to achieve this outcome. It has here been shown that the use of such a product increases recipient survival, improves host responsiveness to glucose, and dramatically reduces the immunological challenge to the graft. Furthermore, the incorporation of immune effectors such as Sertoli cells demonstrate the potential of providing an added level of support to transplanted tissue by providing factors key to the maintenance and protection of the islet graft.

Despite the various successes with islet encapsulation, there remain a number of shortcomings to the therapeutic potential of this therapy. Foremost among these is the inconsistency from center to center with respect to the alginate used and the purification

regimes used, if any. Ideally, improved sharing of information on the alginate used and the methods employed to extract, modify, and purify the product might alleviate much of this problem. Also, improved understanding of the factors mediating graft failure could be key to the development of better capsules. In reality, however, it is fundamental to understand that the capsule alone lacks the ability to provide complete protection of the graft from host immune effector molecules. It is suspect that the capsule will ever alone have the ability to completely address the problem of antigen shedding from the capsule, or the subsequent immune activation. The most striking example of this would be the release of insulin from the capsule, which in itself has been shown to have immune consequence in type 1 diabetics (33). As such, additional techniques must be employed to prevent antigen detection, reduce macrophage activation, inhibit CD4+ T cell-activation, and so on. The use of Sertoli cell may provide a level of support in this regard, though the determination of the factors released from Sertoli cell affecting immune function, and the mechanism by which these occur, needs to be determined to maximize their therapeutic potential. As these various concerns are addressed, the true therapeutic potential of encapsulated islets will become clearer, and the future of encapsulated islet transplantation will be evident.

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# APPENDIX A

# ENCAPSULATION DOES NOT PROVIDE LONG TERM SURVIVAL OF MOUSE ISLETS IN ALLOGENEIC RECIPIENTS

## A.1 OBJECTIVE

To determine whether or not mouse islets encapsulated in a purified alginate microcapsule have the ability to survive and function when transplanted into allogeneic recipients.

## A.2 RATIONALE

In Chapter 2 we demonstrated the ability of the alginate microcapsule to prolong survival of islet grafts in immune compromised animals. In Chapter 3 we demonstrated the ability of a modified, improved alginate to improve the longevity of graft survival and function using syngeneic mouse islets. As a consequence of these successes, the next obvious step was to transplant encapsulated islets into same species recipients of increased immunologic challenge, namely allogeneic recipients. A number of techniques have been employed in an attempt to prolong allogeneic allograft survival in small animal models, such as the use of pre-transplant immune modulation of islets (1), immunosuppressants (2;3), monoclonal antibodies (4), chimerism/tolerance induction (5) and the transplantation of islets into immune privileged sites (6), among others. Here we

investigate the use of alginate encapsulation as a means of protecting allogeneic donor islets in an immune competent recipient. As the capsule has the ability to block cell-cell contact between the donor antigen presenting cells and host immune cells, the belief was that the encapsulation of islets in an immunologically inert alginate capsule would have the ability to protect the transplanted islets, particularly from the direct antigen recognition and subsequent destruction of the graft characterized in allograft rejection (7).

## A.3 EXPERIMENTAL PROCEDURE

BALB/c mouse islets were isolated and encapsulated in 0.75% (w/v) alginate purified in house from Pronova UP-MVG alginate (high G alginate, batch #701-256-08; NovaMatrix, FMC Biopolymer, Drammen, Norway) dissolved in HBSS (without Ca++, Mg++) (pH 7.4) as previously described (Chapter 3). Grafts of approximately 1000 islets were implanted into the peritoneum of a total of 12 C3H mice with diabetes induced by streptozotocin (STZ; 225mg/kg body weight, freshly dissolved in acetate buffer, pH4.5; Sigma Chemical Co., St. Louis, MO) injection 3 days prior. Two BALB/c mice were made diabetic (STZ; 275mg/kg body weight), transplanted with encapsulated BALB/c islets and served as controls. Blood glucose was monitored over time until animals returned to hyperglycemia or until the end of the experiment (Day 49), when capsules were recovered and observed for gross morphology of the capsules, for the condition of the recipient peritoneum, and for immunohistological examination. Data are expressed as average ± the standard error of the mean (SEM).

## A.4 RESULTS

When we transplanted encapsulated BALB/c islets into diabetic C3H recipients one of the animals died following surgery. The rest of these mice demonstrated an almost immediate return to normoglycemia (blood glucose ≤8.7mmol/l) (Figure A-1). Nine of the eleven remaining recipients showed a dramatic rise in blood glucose levels following one week post-transplantation, with a return to hyperglycemic blood glucose levels (≥17.8mmol/l) averaging at 22.6±3.2 days for all allogeneic recipients. Of the two BALB/c mice transplanted, one was sickly and required termination 24 hours following transplantation. The other syngeneic recipient maintained normal blood glucose levels to termination at 28 days (Figure A-1).

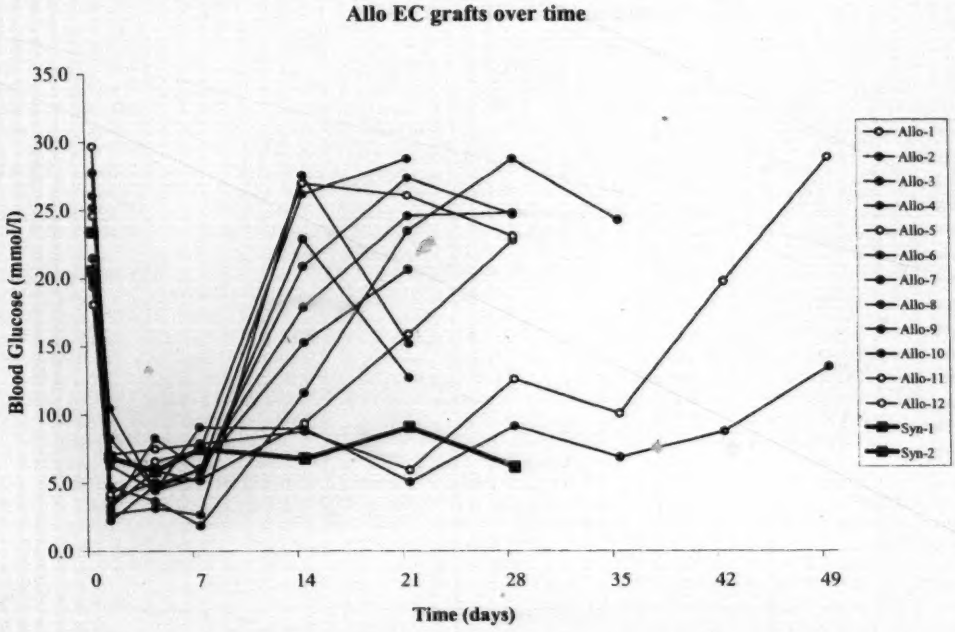


**Figure A-1.** Blood glucose levels over time of mice transplanted with encapsulated BALB/c islets. Controls are denoted via thicker lines.

When grafts were recovered from C3H mice, 6/11 (55%) demonstrated swelling of the internal organs, particularly the liver, and 5/11 mice (45%) were found to have their capsules clumped together between the liver and diaphragm. Furthermore, immunohistochemical observation of the recovered capsules revealed positive insulin staining (Figure A-2A) but negative glucagon (Figure A-2B) staining of islets inside the capsule, and with a minimal macrophage (CD11b) staining (Figure A-3A) but negligible CD4+ T-cell (Figure A-3B) and CD8+ T-cell (Figure A-3C) staining surrounding the recovered capsule. The surviving BALB/c mouse showed no evidence of peritonitis or liver swelling to 49 days, and capsules remained free floating in the peritoneum.

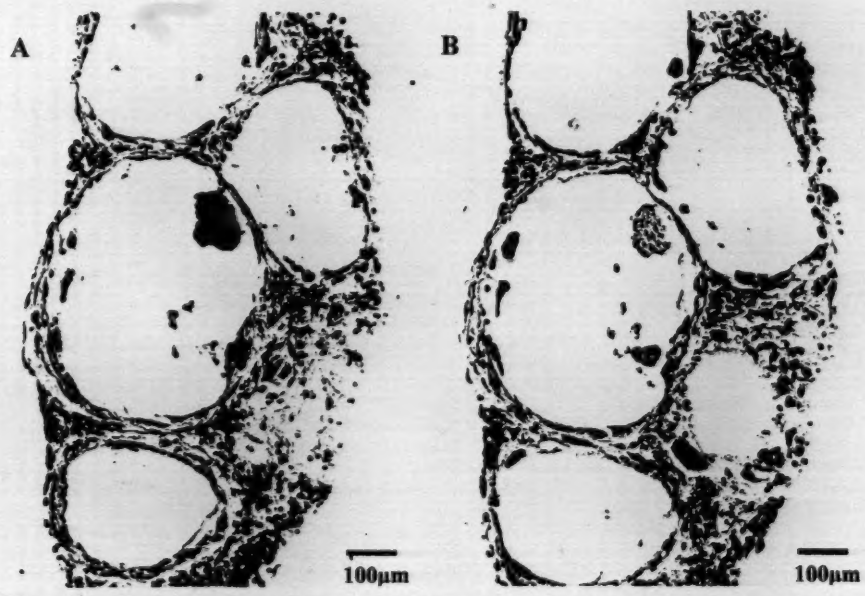


**Figure A-2.** Immunohistochemistry of encapsulated islets recovered at 21 days and stained for (A) insulin and (B) glucagon.

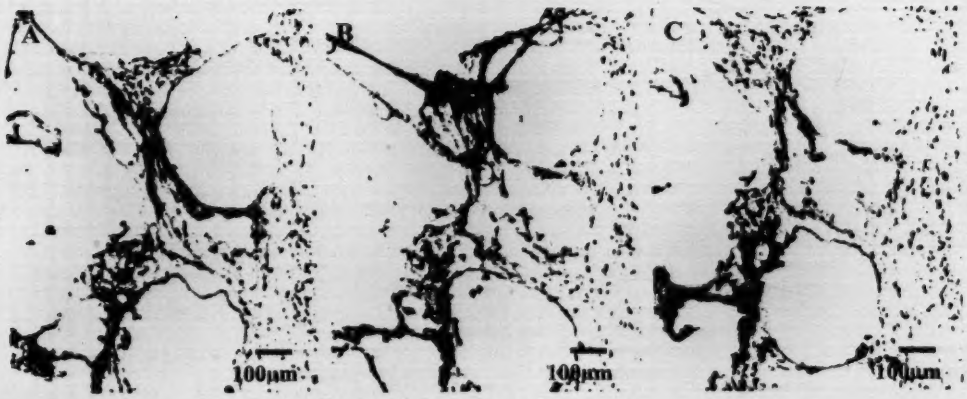


**Figure A-3.** Cryostaining of encapsulated islets recovered at 21 days and stained for (A) CD-11b, (B) CD-4 and (C) CD-8.

## A.5 CONCLUSION

In this experiment, transplantation of encapsulated mouse islets into allogeneic recipients yielded significantly substandard results. We have shown previously that 38 of 42 (90%) of recipients of encapsulated syngeneic islets were able to restore normal blood glucose levels well beyond 100 days post-transplant (Chapter 2). Though all animals were able to alleviate hyperglycemia following transplantation of allogeneic islets, this state was short lived. Furthermore, many recipients exhibited lower than normal physiological levels of glucose in their blood, indicating perhaps that cell death and subsequent insulin dumping from the transplanted islets may have occurred. However, observation of the recovered islets demonstrated strong insulin staining with no overt signs of loss of the islets. Furthermore, the mechanisms of any cell death remain uncertain, staining for immune effector cells indicated that, though there was some indication of macrophages, the proposed mediators of encapsulated islet cell rejection (9), though there was virtually no sign of CD4+ T-cells(8), indicated in perpetuating the immune response. Such flaws in

the past have been attributed to deficiencies in capsule mechanics (10) or to the bio-incompatibility of the alginate used (11). We feel, however, that deficiencies here lie not in the alginate, but in the inability of a simple, open-pore capsule to fully protect the transplanted allogeneic islets (12-14). As direct mechanisms can be ruled out, a more thorough investigation of the involvement of humoral-mediated and innate immunity are required. Furthermore, to better elucidate the mechanisms of rejection in an allogeneic model, testing for success of function of encapsulated islets transplanted into specifically defined knock-out or transgenic mice might be employed. Regardless, it would seem consistent with other studies to conclude that the capsule alone is insufficient as an immune barrier to fully protect the islets from rejection or loss of function, and that a further reduction of donor immunogenicity, or the additional inhibition of host immunity, is required for transplant success.

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